Medullary Bone and Avian Osteoporosis

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I declare that this thesis has been composed by me and that the work is my own.

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ABSTRACT

Medullary bone is a type of woven bone birds produce in response to oestrogen. It acts as a mineral reservoir for the calcium demands of egg shell formation.The morphology and distribution of medullary bone in the modern laying hen at different stages in its life was investigated and described.

Modern commercial laying hens suffer from osteoporosis (structural bone loss), leading to bone fractures.The association between structural bone loss and medullary bone modelling and remodelling was investigated in three further studies. Bone samples were processed for examination with light microscopy and ultrastructurally. Histomorphometric techniques were used to quantify cancellous, cortical, and medullary bone volumes in undecalcified sections of samples collected from three studies.

In the first of these studies, female fowl were killed either during ovarian follicular development, after laying a single egg, or half way through the laying cycle. Structural bone volume decreased significantly during both medullary bone modelling and subsequent remodelling. Medullary bone volume increased significantly during the same period.

In the second study, medullary bone modelling was induced in male fowl by the administration of oestrogen, and prevented in female fowl by tamoxifen. Oestradiol-treated males had significantly lower structural bone volumes than control males, while tamoxifen-treated females had significantly higher structural bone volumes than control females.

The final study determined the effects of the bisphosphonate alendronate on the structural bone loss associated with medullary bone modelling and remodelling. Alendronate administered before follicular development resulted in significantly greater structural bone volumes both at the onset of lay and at mid-lay than in vehicle-treated controls.

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Bone, the principal calcified tissue of vertebrates, is composed of a collagen fibre matrix, a non-collagenous protein cement, crystals of a calcium-phosphate complex and usually cells called osteocytes. The proportions of each of these can vary, as can the size and arrangement of the cells and fibre bundles, producing different types of bone. In the living organism, bone is continually being formed by osteoblasts, and resorbed by osteoclasts. Weidenreich (1930) classified bone into 5 different types according to fibre arrangement and thickness, but these are difficult to determine histologically and it is more usual to describe bone types as bundle, woven, or fine-fibred (Pritchard, 1972). Bundle bone is made up of regularly arranged coarse fibre bundles (which are visible under the light microscope), with osteocytes which lie in columns between them. This type of bone is found extensively in the skeletons of lower vertebrates and at the attachment of ligaments and tendons in mammals and birds. Woven bone is very rich in large randomly packed osteocytes, and has coarse variable sized fibres which are highly irregular in orientation. It is highly vascularized and mineralised. Both bundle and woven bone are associated with rapid bone formation although the former develops in an orderly manner compared with the more haphazard and erratic formation of the latter. Woven bone can be considered in birds and mammals to be a mainly temporary tissue found, for example, after fracture or during pathological disturbance. It is later replaced by the more permanent fine-fibred bone. Fine-fibred bone, also referred to as lamellar bone, is the principal bone type in mammals and birds. It has relatively small, uniformly ovoid osteocytes which are widely spaced in the bone matrix. Under polarized light, most fine-fibred bone exhibits a lamellar structure. It is formed slowly, in sheets or lamellae, as a lining to vascular channels in existing bone or calcified cartilage, or as a narrow surface layer on existing bone in the adult skeleton. Fine-fibred bone can be divided further into compact and cancellous bone.

Compact bone

Compact bone is the type of bone characteristic of the cortex and is therefore also known as cortical bone. It may be formed in three ways; 1) as cylinders of new bone (primary osteones)

in the vascular channels of fine cancellous membrane bone or cartilage; 2) as primary solid bone on periosteal or medullary surfaces of existing cortices; or 3) as secondary osteones (Haversian systems) as a result of cortical bone remodelling. These different formation processes produce the three recognisable types of compact bone (Smith, 1960). The proportions of each bone type are dependent on the age, size, and species. In large mammals primary osteonal bone is typical of younger animals, being replaced by secondary osteones with advancing age. Dinosaur bones also contain numerous secondary osteones. In contrast, smaller mammals such as mice and rats do not normally have secondary osteones, and neither do birds as large as the albatross, according to some authors (von Eggeling,1938; Cohen & Harris, 1958). However, the bone of young growing birds has been reported by other authors to comprise primary osteones which in adults are replaced by secondary osteones (Foote, 1916; Enlow & Brown, 1956). In birds and mammals the general rule is that Haversian remodelling is related to size; for example, small birds have thin cortices which are not remodelled while domestic fowl and ostriches have numerous Haversian systems (Belanger & Copp, 1972; Currey, 1984; Rubin & Lanyon, 1988).

Osteones are built around blood vessels, with their long axis mainly parallel to the long axis of the bone. In transverse section they appear as approximately circular profiles called Haversian systems. The osteocytes are regularly arranged and orientated around the vascular canal within the osteones and their canaliculi extend radially and anastomose with those of neighbouring osteocytes. Primary osteonal systems are small, with no definite boundary, each system blending into the next. Their central canal may contain several vessels. Secondary osteonal systems are larger, contain a single vessel, and have a definite boundary marked by a cement line. Interstital bone occurs between individual secondary osteones and comprises either of the remains of remodelled osteones or the blind ends of other osteone branches (Pritchard, 1972).

Cancellous bone

The presence of cancellous bone is widespread throughout the skeleton, forming the bulk of the bony substance within the vertebrae, the cranium, and in the ends of the long bones, for example. It consists of interconnecting fine bony trabeculae whose spaces contain marrow.

Each trabecula is composed of fine bony lamellae interspersed with osteocytes and contains numerous cement lines indicative of previous resorption and formation. Occasional fragments of calcified cartilage which have escaped remodelling may be embedded in the bone. The bone surfaces are covered by a single layer of bone lining cells, interrupted in places by active osteoblasts and osteoclasts (Pritchard, 1972; Hodges,1974).

These different bony elements, organised into a skeleton, have a dual function; they must provide mechanical support for the animal's tissues and also play a major role in mineral homeostasis. These functions are maintained by the bone cells, which are active in the perpetual modelling and remodelling of bone.

Bone cells

Osteoblasts:

The structure of the osteoblast is a reflection of its current function; the synthesis of the collagen matrix of bone and its subsequent mineralisation (Holtrop, 1990). The active osteoblast has a large, eccentrically located nucleus containing (in section), one to three well developed nucleoli. Rough endoplasmic reticulum occupies another major compartment of the active osteoblast, separating in some places to form cisternae. The Golgi apparatus is prominent, and is concerned with the synthesis and discharge of collagen in association with secretory granules. Mitochondria can be found throughout the cytoplasm and occasionally contain electron dense particles which have been demonstrated to have a high calcium and phosphorus content. As bone formation progresses, the osteoblasts at the formation site decrease in number, become more flattened, formation slows down, and the osteoblasts become bone lining cells or osteocytes (Recker, 1992).

Bone lining cells:

These are those osteoblasts which, after the remodelling cycle, have not become embedded in the newly formed bone. Only the nucleus of the lining cell, elongated against the bone surface, is visible with light microscopy. Ultrastructurally, they have very little cytoplasm which typically contains small amounts of rough endoplasmic reticulum, free ribosomes and a few mitochondria (Holtrop, 1990). Lining cells are in contact with the osteocytes via the latter's cannaliculi, and are believed to have a role in preparing the bone surface for resorption. Exposure to parathyroid hormone results in the cell contracting and secreting enzymes which remove the persisting thin osteoid layer at the bone surface, which is a prerequisite to bone resorption (McSheehy et al, 1986).

Osteocytes:

Osteocytes are those osteoblasts which have become embedded in newly formed bone. In the transition from osteoblast, newly embedded osteocytes reduce in volume by approximately 30% (Marotti, 1976). This process continues as the osteocyte fills its lacuna with new bone; the smallest osteocytes and lacunae therefore tend to be furthest from the bone surface (Yeager, 1975). The osteocyte in lamellar bone is elongated, orientated with its long axis parallel to the collagen fibres and has multiple cytoplasmic processes extending into canaliculi. These canaliculi form a dense network in the bone matrix and are probably necessary for ion and nutrient exchange between cells (Holtrop, 1990). Osteocyte function has not been definitely established but it has been suggested that they may be sensitive to strain changes during mechanical usage (Lanyon, 1992). Osteocytes are destroyed during bone resorption.

Osteoclasts:

Osteoclasts are large (20-100 μm in diameter) multi-nuclear cells which contain from 2-50 nuclei. They have a 'foamy' cytoplasm containing numerous lysosomal vacuoles, and more mitochondria than any other cell type (Holtrop, 1990). The active osteoclast's most characteristic feature is the ruffled border; these membrane infoldings result in a massively extended interface between the cell and the bone surface, across which exchange can take place. At either end of the ruffled border is a podosome or clear zone containing no organelles but thin filments of an amorphous substance involved in adhesion. Thus a highly acidic

microenvironment conducive to bone resorption is isolated between the osteoclast and the bone surface.

Bone remodelling

The processes of bone formation and resorption occur throughout the life of an animal. The term remodelling is used where old lamellar bone is replaced by new, with little or no change in the mass or shape of the bone (Frost, 1973). Bone remodelling is carried out by an organised group of bone cells collectively referred to as bone remodelling units, whose actions are similar in both cancellous and compact bone. These actions can be separated into 4 phases; activation, resorption, reversal and formation (Frost, 1973; Baron, 1977; Frost, 1986).

Activation is the process that changes a resting surface into a resorbing surface and involves the recruitment and subsequent fusion of the osteoclast precursors, and also the penetration of the bone lining cell layer. In the adult human, a new bone remodelling unit has been shown to be activated every 10 seconds (Parfitt, 1983). The resorption phase is carried out by the newly recruited osteoclast team which, by resorption, create Howship's lacunae in cancellous bone and 'cutting' cones in compact bone. The former are 50μm deep cavities, while the latter are tunnels through the matrix, approximately 2.5μm long and 200μm in diameter (Parfitt, 1983). In the next phase there is a reversal from bone resorption to bone formation, and results in a coupling of resorption to formation. The mechanisms of this coupling are not fully understood, but result in attraction of osteoblasts to the resorption site. The final process of bone formation occurs in 2 stages; firstly the osteoblasts lay down layers of osteoid in the areas previosly excavated by the osteoclasts. These osteoid lamellae are later, and more slowly, mineralised. This occurs after a lag time of approximately 20 days in which the matrix undergoes biochemical changes which make it receptive to mineralisation. Primary mineralisation occurs rapidly, so that within a few days 75% of the final level is achieved. Secondary mineralisation is then completed more slowly, and can take as long as a year (Amprino & Engstrom, 1952). Each complete remodelling cycle carried out by these bone

remodelling units takes approximately six months (1 for resorption and 5 for formation) in trabecular bone from normal women, and slightly longer in compact bone (Recker et al, 1988). Remodelling is necessary for both maintenance of the skeleton's mechanical competence and for mineral homeostasis (Lacroix, 1971; Frost, 1973; Parfitt, 1983).

The mechanical role of the skeleton

The mechanical role of the skeleton has been the subject of many studies, and effects of size, morphology, and bone components on strength have been investigated throughout the last 300 years. Possibly the earliest theoretical assesment of bone structure was made by Galileo in 1638, who concluded that a hollow cylinder was stronger than a solid rod (Bell, 1959, 1969). The mechanical function of cancellous bone trabeculae also interested many early researchers; in 1892 Ward compared the architectural features of the femoral neck to a triangular bracket supporting a street lamp, and earlier comparisons to the pressure and tension lines in a crane were made by Meyer and Culmann in 1867 (Murray, 1936). Later studies investigated bone as a material, and demonstrated its unique mechanical properties; bone has the tensile strength of cast iron but is three times lighter and ten times more flexible (Ascenzi & Bell, 1972). This is achieved through the composite nature of the material; collagen molecules are packed end to end into fibrils, their junctions forming pores into which half of the hydroxyapatite is deposited. The remaining mineral is located along the length and between the fibres (Veis & Sabsay, 1987). The arrangement of the collagen fibres results in the femur, for example, withstanding greater force longitudinaly than transversely, and also in compression rather than tension. Thus the femur is perfectly designed to withstand the forces applied to it during weight bearing and locomotion (Reilly & Burstein, 1975).

Although 70-80% of the variance in the ultimate strength of bone is accounted for by bone density (Smith & Smith, 1976), this can be offset by changes in material composition or structural geometry (Einhorn, 1992). In order for the mechanical competence of the skeleton to be maintained, the morphology and mass of bone must be adjusted and regulated (Lanyon, 1992). Remodelling is necessary for the repair of microdamage incurred through normal

loading (Frost, 1986), but is also increased in response to increased loading of bone (Hert et al 1972; Churches & Howlett, 1981; Bouvier & Hylander,1981).

The Mineral Homeostatic Role of the Skeleton

99% of the body's calcium content is located in the skeleton, along with 35% of its sodium, 80% of its carbonate and citrate, and 60% of its magnesium (Martin et al, 1987). This mineral reservoir is particularly important during deficiency states, when it may be drawn upon in conjunction with other mechanisms to rectify imbalances. For example, a drop in plasma calcium concentrations results in increased secretion of parathyroid hormone, which can mobilise calcium by three methods; 1) increased uptake from the gut, 2) increased tubular reabsorption from the kidney, and 3) increased activation of new bone remodelling units. Although the remodelling response is slowest and least sensitive of these, it is the most important for long term homeostasis, being virtually unlimited in capacity (Rasmussen, 1961; Martin et al, 1987). Also, the nature of the remodelling unit allows for calcium to be mobilised fairly quickly and replaced over a longer period of time (Parfitt, 1981).

The role of remodelling in calcium homeostasis is of paramount importance during egg-laying in birds, antler formation in deer, and pregnancy and lactation in mammals (Banks et al, 1968; Dacke, 1979; Parfitt, 1981; Feinblatt, 1982; Miller et al, 1986; Miller et al, 1989). In large animals, such physiological calcium stresses generally result in increased remodelling in compact bone rather than cancellous bone in order that large amounts of calcium can be mobilised with minimum impact on skeletal integrity (Parfitt, 1981; Parfitt, 1988). This suggests that different bone envelopes differ in their sensitivity or response to different levels of calcium stress.

The Avian Skeleton

In general, the structure of bone and the mechanisms of bone turnover in birds are similar to mammals (Meister, 1951; Hodges, 1974; Loveridge et al 1992). However, the avian skeleton

has some adaptions related to egg-laying and flight.

Flight

All birds, including those domesticated species which have become terrestrial, possess a skeleton which has been adapted for flight (Feduccia, 1975). One such adaptation is a general reduction in the weight of the skeleton (King & King, 1979). The skeleton of the pigeon has been found to be 4.4% of the total body weight, compared with 5.6% in the rat (Welty, 1962). Hildebrand (1972) recorded the skeleton of an eagle to be less than 7% of the total body weight, and estimated that this was half the value for man. Measurements of bone density in more than 20 species of bird and several species of mammal showed the femur, tibia, ulna and radius to be 10-15% less dense in birds than in mammals (Chappel, 1978). This reduction in weight and density can be attributed to both the reduction in cortical thickness typical of birds and to the phenomenon of pneumatisation.

In birds, many of the bones of the skull and the post cranial skeleton are hollow and contain air sacs which are bounded by epithelium continuous with the respiratory system (Bellairs and Jenkin, 1979). Different species are pneumatized to varying degrees, but in the domestic fowl most of the cervical vertebrae, the fifth thoracic vertebra, the second and third sternal ribs, the first two vertebral ribs, the lumbosacral mass, pelvic girdle, parts of the sternum and coracoid, and the skull and humerus are all pneumatised (King, 1957). The cavity of a pneumatised bone has a relatively greater volume and decreased bone density than the corresponding non-pneumatised bone of another species. It also has a greater circumference but thinner cortex, giving it a greater resistance to bending strains. Thus the lightening of the skeleton to aid flight is acheived without compromising strength (Bellairs and Jenkin, 1979). Pneumaticity does not of course have a purely mechanical significance; it also has a role in respiration and temperature regulation.

A further adaptation in bird skeletons is the fusion and concomitant deletion of some bones, which confers strength and rigidity (Huxley, 1871; Feduccia, 1975). The cranium and pelvic girdle are the most obvious areas of fusion, but fusion and deletion occur extensively in the

appendicular skeleton. In the wing, the carpals, metacarpals and phalanges exhibit fusion, while the tarsals and metatarsals are fused in the leg.

Egg laying

Calcium demand and turnover in egg-laying birds is extraordinary when compared with all other classes of vertebrate. In the domestic fowl each egg shell contains about 2.3g calcium, which is equivalent to approximately 10% of the animal's total body calcium (Etches, 1987), and a similar proportion is found in other birds. This represents a calcium demand of 1.0g calcium kg body wt/day (Miller, 1992). In contrast egg-laying reptiles, e.g. the green turtle (*Chelonia mydas*), mobilize 0.14g/kg body wt/d calcium to meet the requirements of egg-shell formation (Simkiss, 1961). In laying hens, the progress of the egg down the reproductive tract, from ovulation to oviposition, ranges from 24-28 hours in duration. The lengthiest part is the period in the shell gland, which lasts between 20 and 26 hours . A 3 hour period of "plumping" is followed by shell-formation, which accounts for 15 hours, and the remaining time is taken up by shell pigmentation. During shell formation the shell gland secretes 100-150mg calcium/hour, and at this rate circulating blood calcium would be depleted in less than 18 minutes, if it was not replenished by increased intestinal absorption and skeletal mobilisation (Johnson, 1981). Intestinal absorption of calcium is greatly increased with the onset of reproductive activity, due to increased renal production of 1-α-hydroxylase. This is required for the conversion of $25(OH)D_3$ to $1,25(OH_2)D_3$ (Castillo et al, 1977), on which 70% of the bird's calcium absorption is dependant (Hurwitz, 1992). However, only 60-75% of calcium in the shell is derived from dietary sources, the remainder (0.57-0.92g/egg) being mobilised from bone (Driggers and Comar, 1949). This represents a massive and potentially harmful depletion of the bird's skeletal calcium stores, and as a result they have developed a unique mineral reservoir system within their bones called medullary bone.

Medullary Bone

Medullary bone was first observed during a 2 year study of 449 male and 401 female pigeons (Kyes & Potter, 1934). The males were unaffected and the medullary cavity of their long bones was completely filled with red marrow. While half of the females killed at random were

similar to the males, in the remainder, the marrow of the long bones of the legs was ossified to varying degrees. Histological examination revealed delicate spicules of bone extending from the inner surface of the shaft a short distance into the marrow in some cases, and in others anastomosing trabeculae of bone extended in every direction throughout the medulla into a grey marrow. The degree of marrow ossification in female pigeons was directly related to the development of the ovarian follicles; those with a follicle 10mm in diameter were extremely ossified, a follicle > 4.5mm slightly ossified, and those with follicles less than 2mm in diameter showed no marrow ossification.

Later studies confirmed the presence of medullary bone in reproductively active female domestic fowl, geese, ducks, sparrows, canaries, and quail (Pfeiffer et al, 1941; Landauer et al, 1941; Bloom & Domm, 1941; Bloom et al, 1941; Bloom et al, 1942; Common et al, 1948; Simkiss, 1961). The formation of medullary bone explained an earlier finding that hens enter into a positive calcium and phosphorus balance approximately 10 days prior to lay, so that they retained more calcium than was excreted (Common, 1933).

The first detailed histological study of medullary bone formation in the domestic fowl was by Bloom et al (1958). Mature cockerels were reported not to have medullary bone; haematopoietic marrow and fat cells filled the marrow cavity and the endosteal surfaces of the cortices were covered by spindle-shaped lining cells. In contrast, changes occurred in the long bones of the females during development of the ovarian follicles; in the early stages of follicular development short trabeculae of medullary bone began to form on the endosteal surfaces of the cortices. These were composed partially of osteoid and were covered by a layer of osteoblasts, but osteoclasts were scarce or absent. By the later stages of follicular development, a dense network of medullary bone with broad and frequently interconnected trabeculae extended from the cortex half way through the marrow. Once egg laying was established, a sequence of rapid medullary bone formation and resorption was observed which closely followed the formation of the egg shell.

Medullary bone develops as a result of the synergistic action of androgen and increasing

levels of oestrogen produced by the developing ovarian follicles as point of lay approaches (Taylor & Stringer, 1965). In pigeons and chickens, both androgens and oestrogen are necessary for medullary bone formation (Bloom et al, 1942; Taylor et al, 1971), and sexually mature male birds can be induced to form medullary bone by the administration of oestrogen (Bloom et al, 1942; Landauer & Zondek, 1944) Although medullary bone formation is initiated by oestrogen, it is not clear whether its action is direct or indirect (Schraer & Hunter, 1985). However, Oshashi et al (1990) have suggested that medullary bone osteoblasts are oestrogen target cells in birds.

The mineral phase of medullary bone is chemically similar to that of compact and cancellous bone, but the apatite crystals are distributed differently throughout the matrix. In many areas, the hydroxyapatite crystals are randomly orientated in respect to the direction of the collagen fibrils, and their periodic banding. (Ascenzi et al, 1963). In lamellar bone, hydroxyapatite crystals are predominantly distributed at the level of the A-band of the collagen fibrils. It is also more highly mineralised (Taylor et al, 1971) and has little apparent lamellar organisation of the collagenous filaments (Miller, 1992). The orientation of the medullary bone trabeculae also appear random (unlike cancellous bone trabeculae), and these characteristics are typical of woven bone (Schraer & Hunter, 1985) and are consistent with medullary bone having a role in mineral homeostasis rather than a structural function (Miller, 1992).

Medullary bone remodelling and the egg laying cycle

Since Bloom's observation (1958) that during shell calcification, vast numbers of osteoblasts and osteoclasts were associated with medullary bone, the latter's role in mineral homeostasis has been confirmed. Although medullary bone osteoclast numbers have been shown to be highly variable between individuals (Bloom et al, 1958; Taylor & Belanger, 1969; Miller, 1977), several histomorphometric and ultrastructural studies have indicated that their activity is greatly increased during the period of shell formation. In quail and chickens, the active osteoclastic bone surface is considerably increased during shell formation (van de Velde et al, 1984), and osteoclasts spread over the bone, developing ruffled borders, as shell calcification begins (Miller, 1977; 1981). Outwith the period of shell calcification, the osteoclast ruffled

border disappears and the cell becomes rounded and unattached to the bone surface. This period of increased osteoclastic activity is accompanied by a corresponding increase in osteoblastic activity (van de Velde et al 1984). Studies using a radiolabelled tracer (van de Velde et al, 1985) indicated that while matrix formation occurs at the same time as resorption, its mineralisation is delayed until after oviposition but before formation of the next egg (Figure 1.). These results correlate with changes in plasma enzymes; plasma acid and alkaline phosphatase levels peak during shell calcification, but alkaline phosphatase levels drop before shell completion, while acid phosphatase remains high until shortly after oviposition (Taylor et al, 1965).

Medullary bone in female birds is unique in that its role is entirely one of mineral homeostasis, and it appears to have no structural function. The medullary bone deposits apparently allow the animal to store a considerable amount of readily available calcium for shell formation without compromising the structural skeleton (Miller, 1992). Medullary bone is then rapidly resorbed when follicular activity ceases (Wilson et al, 1991). In birds which are seasonal layers, or which are permitted to incubate their eggs, medullary bone is therefore a temporary tissue.

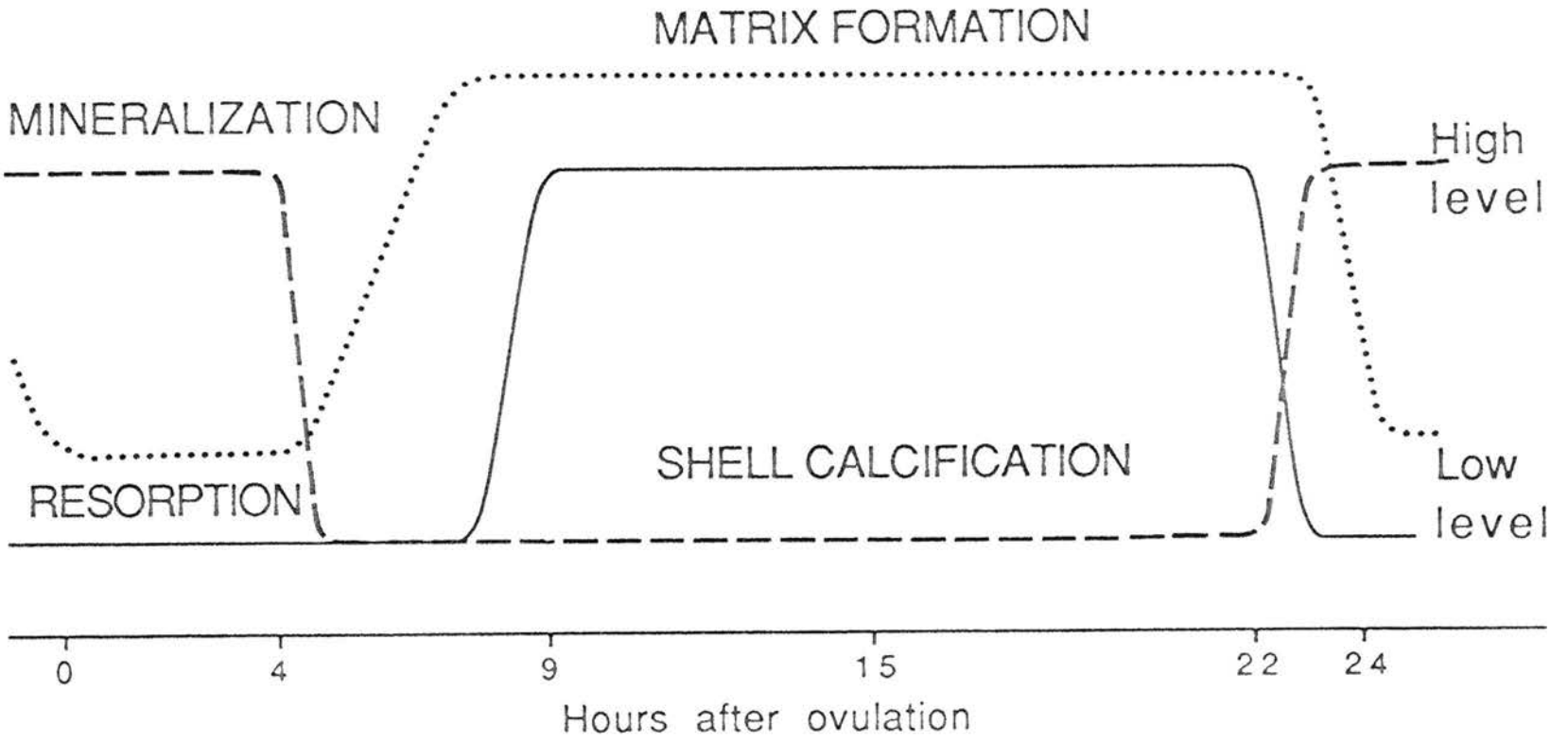


Fig. 1 Model of the processes of resorption, matrix formation, and

The Red Junglefowl (*Gallus gallus*) is a sub-tropical and equatorial species. In the north of its natural habitat it is a long-day breeder and in equatorial regions either a dry-seasonal or no seasonal pattern breeder. A flock of wild jungle fowl normally comprises a male and one to seven females who lay clutches of between five and seven eggs (Nishida, 1980). It was domesticated in the Indus region around 2000 B.C., and provided a permanent supply of flesh, eggs, grease and feathers (Wood-Gush, 1959). The domesticated fowl was already present in Britain when the Romans invaded around 50 B.C., and probably remained virtually unchanged for the next 1,900 years. This century, the domestic fowl has undergone dramatic changes through selection and environmental manipulation for commercial gain. Frequency of oviposition (egg-laying) was increased by provision of shelter against predators and fear-inducing disturbance (Romanoff & Romanoff, 1949), manipulation of daylength by artificial illumination reduced the age of sexual maturity and extended the period of gonadal activation (Farner, 1966), and egg numbers were increased by egg removal, and preventing incubation (Laven, 1940). Meanwhile, selection for early onset of sexual maturity, and strains which maintain mature gonads throughout the year have markedly increased the duration of the egg laying cycle from only a few weeks in the Red Junglefowl to approximately fifty weeks in commercial egg-laying hens by the early 1980's (Sossinska, 1982).

Recent improved knowledge of poultry genetics, diseases, nutrition, general management, and physiology has been combined to result in remarkable improvements in productivity in the last 50 years. Some 'primitive' breeds of domestic fowl produce a clutch of 12-14 eggs then cease for a broody period of approximately 2 weeks before the next clutch begins (Harland, 1927). In contrast, the modern commercial layer's laying period extends to more than 50 weeks during which she may produce in excess of 300 eggs. In the 1950's chickens began to be bred exclusively for either egg or meat production (Phillips et al 1985). In 1939, 98% of British egg production was free-range, from a large number of flocks each consisting of approximately 120 hens. 40% of the nation's egg consumption was imported because producers had not mastered the technique of producing eggs in winter.and average production

was 150 eggs/hen/year By 1992, British egg producers were net exporters and 85% of eggs produced were from battery systems. Average egg production was 250 eggs/hen/year. These improvements are continuing; in German random sample tests, egg production in Hisex hens was increased from 271 to 294 between 1981 and 1991. During this time feed conversion was reduced from 2.66 to 2.33, and egg-shell strength increased from 3.30 to 5.30 (Paice, 1993). This effectively means that modern hens are producing more eggs with thicker shells from less feed.

In the modern, commercial egg-laying domestic fowl, medullary bone cannot be considered a temporary tissue, because it is maintained for most of the bird's life. Although some work was carried out more than 40 years ago on the distribution and morphology of medullary bone hens at the start of the laying cycle (Taylor & Moore, 1953), this is probably not entirely relevant to laying hens in production in the 1990's. The aim of this series of introductory histological experiments is to establish the distribution and morphology of medullary bone in the modern layer at different stages in its lifetime.

Samples of bone from the femur, tibiotarsus, tarsometatarsus, phalanx 111 digitus 1 (toe), humerus, radius, ulna, coracoid, clavicle, ribs, and thoracic vertebrae were collected from five laying hens aged 48 weeks. Samples of proximal tarsometatarsus and thoracic vertebrae were collected from commercial layers aged, 30, 50, and 70 weeks.These were trimmed and processed for decalcified and undecalcified sections.

Decalcified sections

Samples were fixed in 10% buffered neutral formalin for 7 days prior to decalcification in Gooding Stewarts Fluid (formaldehyde, formic acid, and distilled water at a ratio of 1:1:8). Samples were washed in tap water overnight, returned to buffered neutral formalin and processed through ascending alcohol concentrations and CNP 30 to paraffin wax. 4μm sections were cut and stained as follows:

Haematoxylin & Eosin

1.) Deparaffinised & hydrated

2.) Stained in haemalum for 5 minutes (solution A [0.03% sodium iodate, 8% aluminium potassium sulphate, 8% chloral hydrate in distilled water] heated gently and mixed with solution B [0.25% haematoxylin in 9% ethanol] at a ratio of 1.66:1. Glacial acetic acid [0.096%] added after 48 hours)

3.) Rinsed in water for 5 minutes

4.) Stained in 2% aqueous eosinY for 2 minutes

5.) Dehydrated, cleared, and mounted in DPX

Verhoeff's Van Gieson

1.) Deparaffinised and hydrated

2.)Stained in a solution containing 40ml 5% alcoholic haematoxylin, 16ml 10% ferric chloride and 16ml of Verhoeff's iodine (2% iodine and 4% potassium iodate) for 15-20 minutes until sections are black.

3.)Rinsed in water for 2 minutes

4.) Differentiated in 2% ferric chloride until the elastic fibres are clearly visible

5.)Rinsed in water

6.) Washed in 95% alcohol to remove iodine staining

7.)Washed in water for 5 minutes

8.) Counterstained with Van Gieson (100ml of saturated aqueous picric acid solution +10 ml 1% aqueous acid fuchsin)

9.) Dehydrated, cleared and mounted

Undecalcified sections

Samples were fixed in 70% ethanol for 10 days then processed on rollers for the production of methylmethacrylate sections according to the following schedule:

1. Defatting solution (chloroform:xylene:ethanol at 4:4:1) for 3 days at room temperature (RT).
2. 100% ethanol for 3 days at RT
3. 50:50 ethanol methylmethacrylate (Aldrich) for 3 days at RT
4. 3 washes methylmethacrylate each for 3 days at RT
5. Impregnation with polymethylmethacrylate (pMMA) for 2 weeks at 4 C.
6. Samples were placed under vacuum for 24 hours at RT.
7. Tissue embedded in fresh pMMA and polymerized at 35 C for 48 hours then 45 C for a further 48 hours.

pMMA was prepared as follows:

1. Equal volumes of methylmethacrylate and 5% sodium hydroxide were added to a separating funnel and shaken vigorously, then the lower NaOH, water and oxidised phenolate-containing fraction discarded. Repeated x 3.
2. This process was repeated once with distilled water, then the solution frozen and subsequently vacuum filtered to remove water.
3. 1% benzoyl peroxide was added to the filtered solution and finally mixed with 40% polymethylmethacrylate, which was dissolved on rollers at RT .

After polymerisation, the blocks were sanded to expose the bone surface. 8μm semi-serial sections were cut from each block using a Reichert-Jung Polycut E and stored dry between filter paper before staining as follows:

Toluidine Blue

1.) Softened in 70% alcohol for 5 minutes

2.) Stained in 1% toludine blue in 5% EDTA for 5 minutes

3.) Washed in distilled water

4.) Blotted on filter paper, cleared and mounted.

Von Kossa

1.) Rinsed in distilled water

2.)Impregnated with 2% aqueous siver nitrate in darkness for 30 minutes

3.)Rinsed in 3 changes distilled water

4.)Reduced in 0.5% aqueous hydroquinone for 1 minute

5.)Rinsed in distilled water

6.)Toned in 0.02% aqueous gold chloride for 10 seconds

7.)Rinsed in distilled water

8.)Fixed in 2% aqueous sodium metabisulphite for 2 minutes

9.)Rinsed in distilled water

10.) Counterstained in Movat's Red (0.08% brilliant crocein and 0.02% acid fuchsin in 0.5% acetic acid) for 3 minutes

11.)Rinsed in distilled water

12.) Dehydrated, cleared and mounted in DPX

Masson Goldner Trichrome

1.) Mordant in 5% Iron Alum for 2 hours

2.) Rinsed in water for 2-3 minutes

3.)Stained in Weigert's haematoxylin for 1 hour

4.) Rinsed in water

5.) Differentiated in 1% acid alcohol for 15 seconds

6.)Rinsed in water

7.)Rinsed in Ammonia Water

8.) Rinsed in water

9.)Stained in Movat's Red

10.)Rinsed in 1% aqueous acetic acid

11.)Differentiated in 5% aqueous phosphotungstic acid until bone colourless and osteoid bright red

12.) Rinsed in 1% aqueous acetic acid

13.)Stained in 1% light green in 1% acetic acid for 5 minutes

14.) Rinsed in 1% aqueous acetic acid

15.)Dehydrated, cleared and mounted

48 WEEK OLD HENS

Examination of the reproductive tracts of these birds indicated that they were all in lay; all ten of the birds displayed a follicular hierarchy and most had soft shelled eggs in the shell-gland.

Femur

The entire length of the femur was filled with large quantities of medullary bone, which was formed both on the surface of the cancellous bone trabeculae and on endocortical surfaces. It also occurred as apparently free spicules in the marrow, unassociated with lamellar bone. There was no apparent variation in the amount of medullary bone present in different areas of the bone. Spicules of medullary bone contained large osteocytes with prominent nuclei, and their surfaces were associated with numerous large osteoclasts and with short rows of osteoblasts. These osteoblasts were either cuboidal or slightly more flattened against the bone surface. No completely flattened bone lining cells of the type observed on cancellous bone were observed on medullary bone trabeculae.

Undecalcified sections demonstrated the presence of very fine seams of unmineralised matrix on the periphery of the medullary bone spicules. This osteoid was apparent only at high magnification and although it varied in amount between individuals none had the depth of osteoid seam which would indicate osteomalacia.

Under polarised light, undecalcified toluidene blue-stained sections demonstrated the differences between cancellous and medullary bone. The cancellous bone trabeculae exhibited the birefringency typical of lamellar bone, while the woven medullary bone did not (Figure 4).

Tibiotarsus

In both decalcified and undecalcified sections medullary bone appeared similar in morphology to that in the femur. However its distribution was slightly different. The proximal portion had similar quantities of medullary bone to the femur but diminished towards the distal extremity. A central core in the diaphysis was observed to be free of medullary bone.

Tarsometatarsus

The diminishing quantities of medullary bone towards the distal extremity and also the medullary bone-free central core observed in the tibiotarsus were also seen in the tarsometatarsus. Additionally, it was noted that very little medulary bone was present in the area immediately adjacent to the proximal extremity. The morphology of the medullary bone in the tarsometatarsus was similar to that described in the femur (Figure 7)

Phalanx 111 digitus 1

The toe did not contain medullary bone (Figure 6).

Humerus

The humerus was pneumatised in nine of the ten birds sampled (Figure 2a). The histological appearance of the medullary bone in the non-pneumatised humerus was similar to that previously described in the femur (Figure 2b). In the pneumatised humeri the amount and distribution of medullary bone appeared to be limited by the presence of the air sac epithelium (Figure 2c). Medullary bone was present either as small spicules on the surface of the cancellous bone trabeculae and immediately under the air sac, or as linings to resorption cavities within cancellous or cortical bone (Figure 3). These resorption cavities were not observed in cancellous bone trabeculae of non-pneumatised bones.

Radius

Medullary bone was present throughout the bone and was evenly distributed. Its morphology was similar to that described in the femur

Ulna

Medullary bone was similar to the radius in distribution and morphology

Thoracic vertebrae

Only one vertebra, the sixth, free thoracic vertebra was pneumatised and was similar in appearance to the pneumatised humeri. In the remaining vertebrae, medullary bone was present throughout the bone and was similar in morphology to the femur (Figure 5).

Ribs

These were filled with large amounts of medullary bone, and the medullary bone trabeculae were tightly packed in the marrow cavity. The coracoid and clavicle were similar to the ribs in the distribution and quantity of medullary bone present.

30 WEEK-OLD COMMERCIAL LAYERS

All of the birds examined were in lay; there was a follicular heirarchy present and many had soft shelled eggs in the shell gland.

The proximal tarsometatarsus of these birds contained moderate amounts of medullary bone which mainly took the form of small spicules on the surface of the cancellous bone trabeculae or endocortical surfaces. Occasionally, small isolated medullary bone spicules were observed which were independent of the lamellar bone surfaces. The medullary bone appeared well mineralised. There was considerable variation between individuals in the amount of medullary bone present.

The free thoracic vertebrae from these birds were all pneumatised.and medullary bone was formed mainly within resorption cavities in the cortices and cancellous bone trabeculae. These resorption cavities were widespread.

50 week-old commercial layers

All of the birds sampled were in lay.

The proximal tarsometatarsus of these birds contained greater amounts of medullary bone than the 30 week old birds, and in some birds there was evidence of massive medullary bone deposition (Figure 8) There was also considerable individual variation in the amount of medullary bone present. The greater quantities of medullary bone were associated with more isolated spicules situated further into the centre of the marrow cavity. The medullary bone was well mineralised.

The free thoracic vertebrae from these birds were all pneumatised and similar in appearance to the 30 week-old birds but the number and extent of resorption cavities was greater. In many places the cortex was excessively thinned and trabecularised.

70 week-old commercial layers

Six of the twenty birds sampled showed no signs of follicular activity and were therefore considered out of lay. The remaining birds were still reproductively active.

Sections of proximal tarsometatarsus from most laying birds were similar to those at 50 weeks but appeared to have more medullary bone. However there were a number of birds which had follicular activity but whose proximal tarsometatarsus contained very little medullary bone. In the birds which had ceased egg-production, medullary bone had been resorbed, but not completely; narrow seams of medullary bone were visible sandwiched between the old cancellous bone trabeculae and new cancellous bone lamellae (Figure 9). This sandwich of woven and lamellar bone was also observed in the cortices.

The free thoracic vertebrae from laying birds were similar to those at 50 weeks, and in out of lay birds the medullary bone had been resorbed but as in the proximal tarsometatarsus, incompletely.

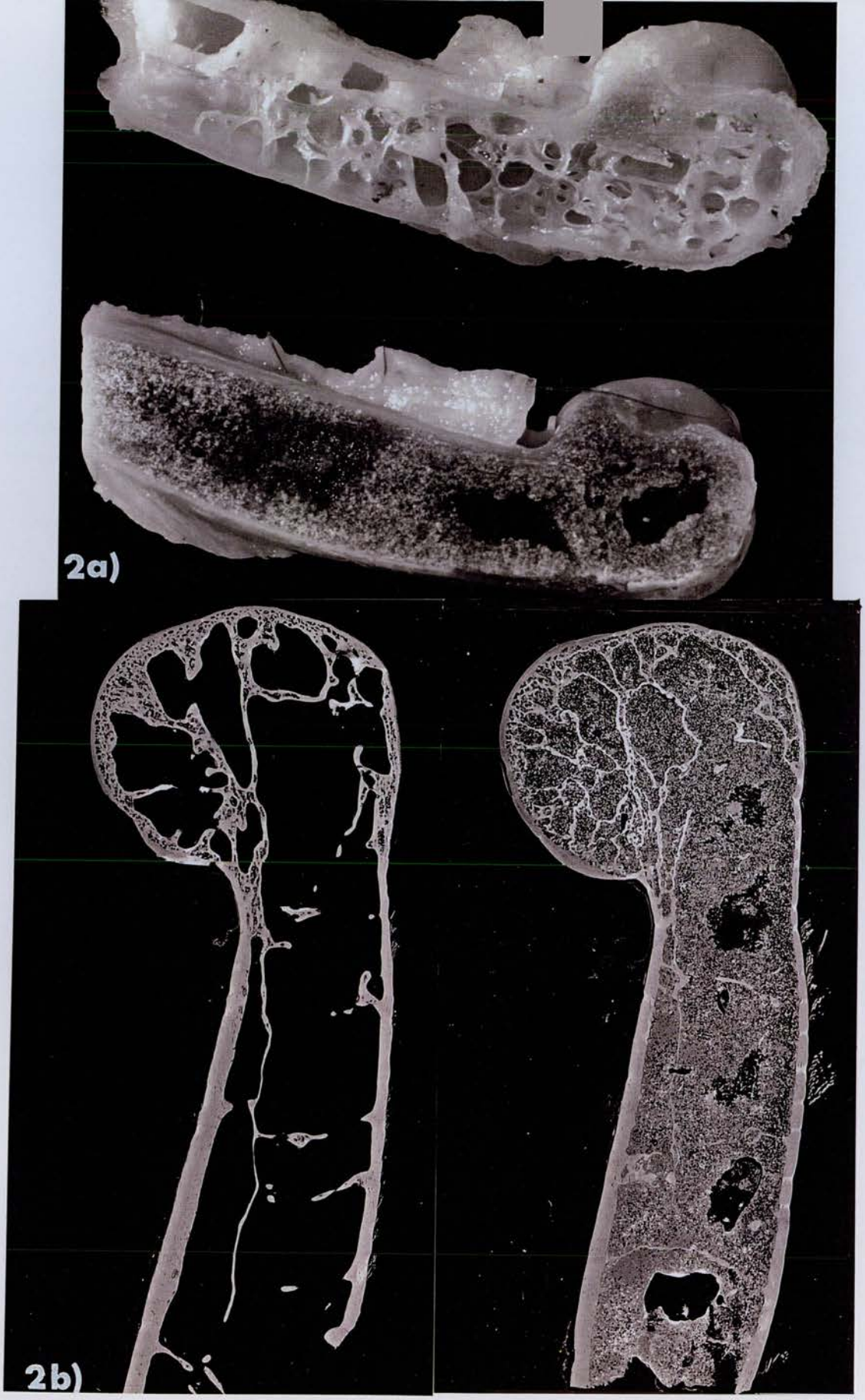


FIGURE 2 : a) Gross appearance of pneumatised (top) and non-pneumatised (bottom) distal humeri (x 6). **b)** decalcified sections of the same bones showing medullary bone trabeculae distributed throughout the marrow in the non-pneumatised humerus (right) and confined to the space between the air sac epithelium and and the cancellous bone surface in the pneumatised humerus (left) (x 7).

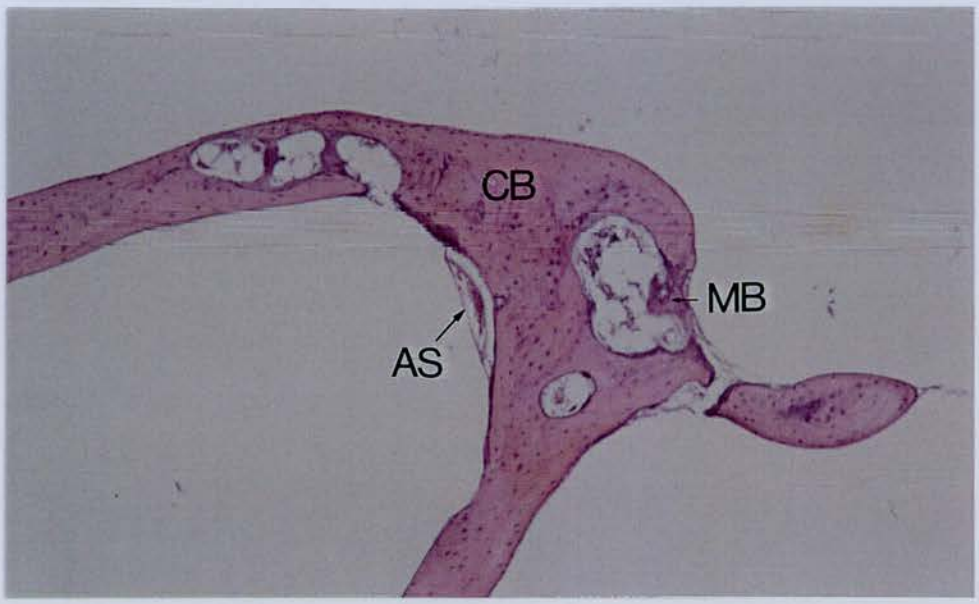


Figure 3. Decalcified section of pneumatised humerus, stained H&E. MB- medullary bone; CB- cancellous bone; AS- air sac epithelium. (x 6.3)

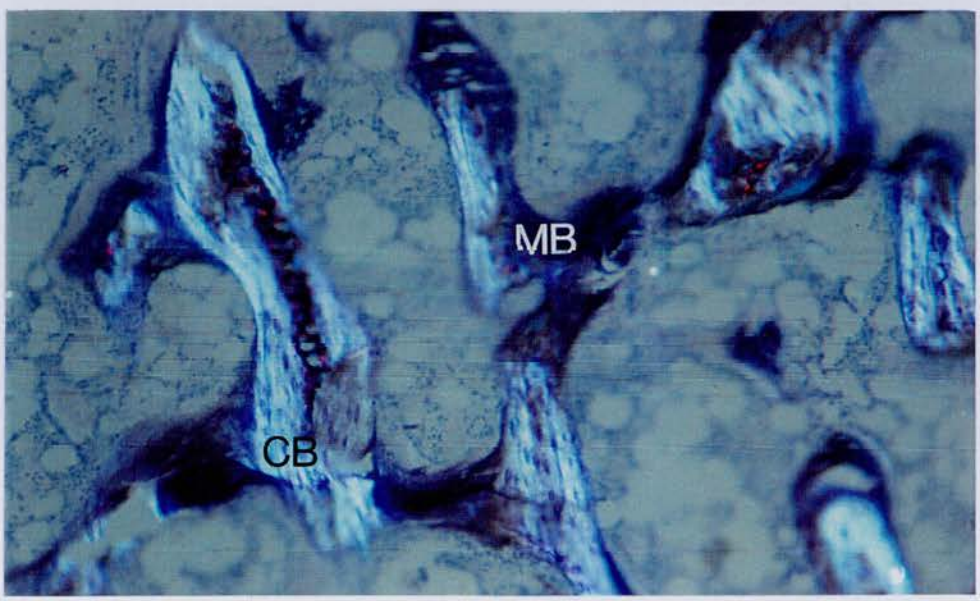


Figure 4. Undecalcified section of femur, stained Tol. blue and photographed under polarised light. Cancellous bone (CB) exhibits its lamellar structure, while medullary bone, because of its woven nature, does not exhibit birefringency (x 12.5)

FIGURE 5 : Undecalcified section of thoracic vertebrae stained MGT; the sixth, free thoracic vertebra is pneumatised (x 10).

FIGURE 6 : Undecalcified section of toe from 48 week-old hen; medullaary bone is absent from both marrow and endosteal surfaces (x 11)

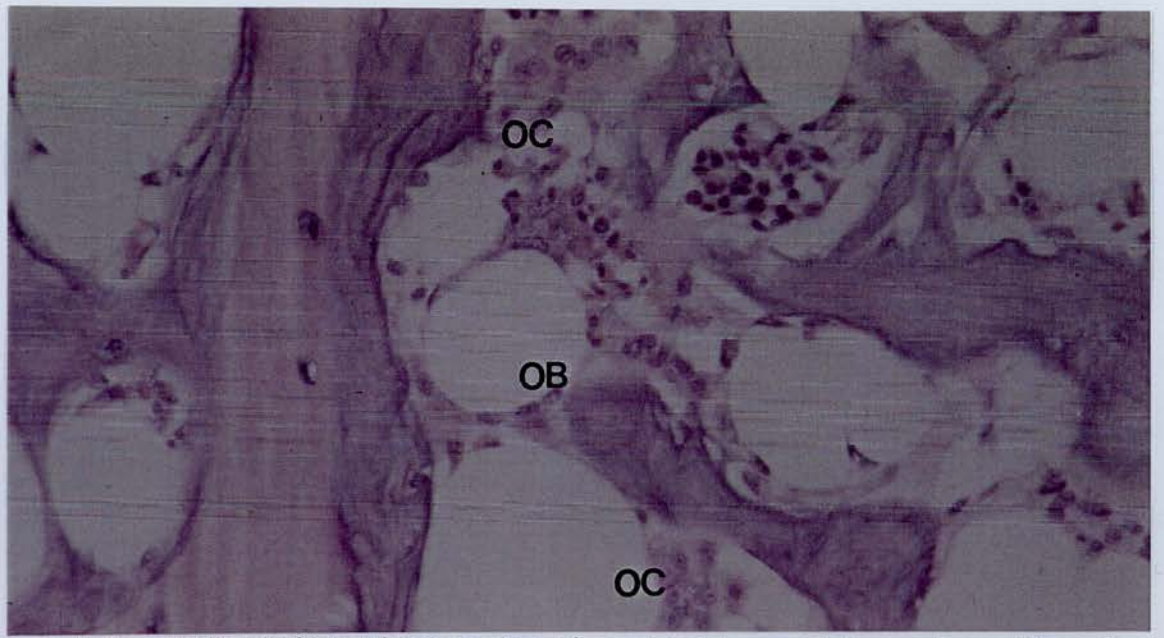


Figure 7 Decalcified H&E stained section of proximal tarsometatarsus from 48 week old hen. Normal medullary bone formation associated with abundant osteoblasts and osteoclasts (x 470)

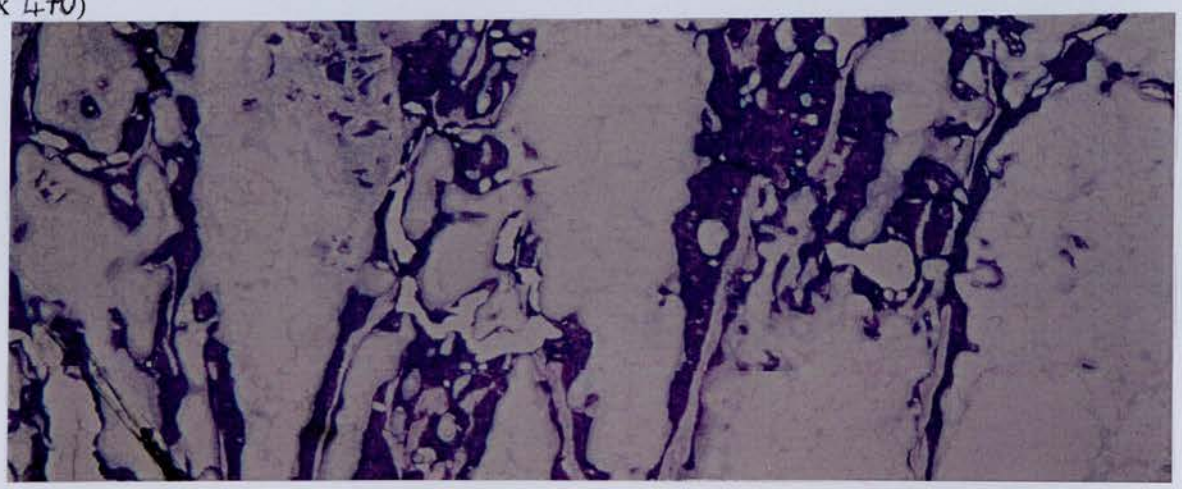


Figure 8 Undecalcified sections of proximal tarsometatarsus stained toluidine blue from 50 week old hen showing massive medulary bone deposition (x 95)

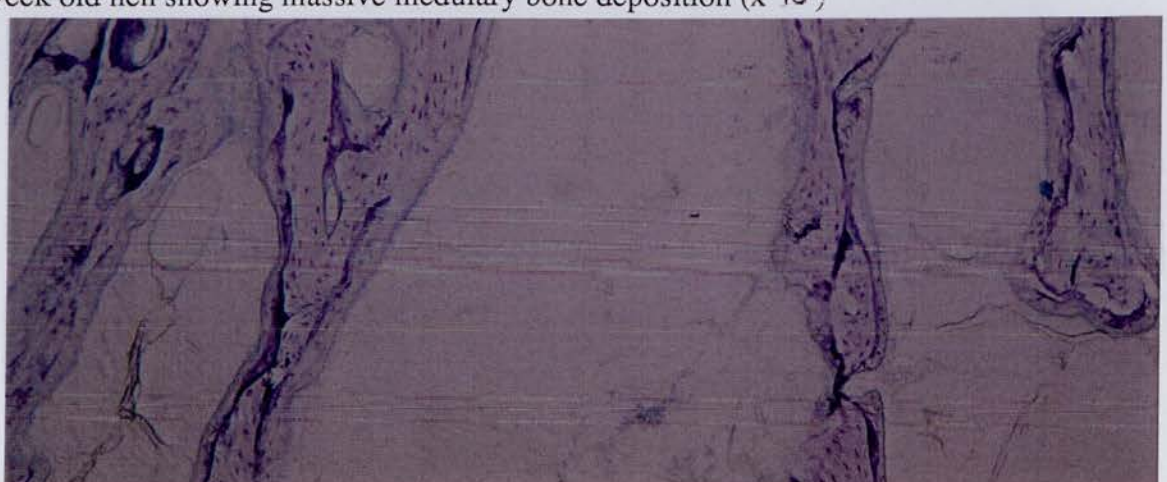


Figure 9 Undecalcified sections of proximal tarsometatarsus stained toluidine blue from 70 week old hen with no follicular activity. Dark staining remnants of medullary bone are sandwiched between cancellos bone lamellae (x240)

The distribution of medullary bone

Although many studies on medullary bone of pigeons, sparrows, ducks and domestic fowl have been carried out (Kyes &Potter, 1934; Pfeiffer et al 1941; Landauer et al 1941; Bloom & Domm, 1941;Bloom et al 1941, 1942; Common et al 1948), these have been concerned mainly with the long bones of the leg. However, Taylor & Moore (1954) attempted to quantify the proportion of medullary bone present throughout the skeleton of the laying fowl at the onset of lay, and reported that medullary bone was present in all bones, but only in very small amounts in the skull, humerus, metacarpals, wing digits, metatarsi and toes. The ribs, ilia, ischia and femur contained the most medullary bone, accounting for between 20.8 and 29.2% of the total dry fat-free weight of the bone. Almost the entire marrow cavity of some bones were described as being filled with medullary bone by the time the hen lays her first egg. 11.7% of the fat-free dry weight of the whole skeleton was medullary bone. These findings were confirmed in a later study (Taylor & Moore, 1956), and it was reported that under conditions of calcium deficiency, mineral was removed from both 'non-labile' (metatarsi and toes) and 'labile' bones (ilia, ischia, ribs). 'Labile' bones were however more severely affected by mineral loss.

The distribution of medullary bone in the present experiment was clearly different in some respects to that observed by Taylor & Moore, (1954, 1956). Although they described the tarsometatarsus as containing little (1954) or no medullary bone (1956), all the tarsometatarsi of hens sampled in the present study contained large amounts of medullary bone. Also, in this experiment, those hens in which the humerus was either partially pneumatised or not pneumatised contained large amounts of medullary bone. However, in agreement with their results, there was definitely variation in this experiment in the volume of medullary bone between different bones; the femur, tibiotarsus, ribs, clavicle and coracoid contained the most medullary bone while those bones which were pneumatised contained considerably less. None of the hens sampled had medullary bone in the toes.

The influence of blood supply on the distribution of medullary bone has also been

investigated by augmenting by fracture the blood supply to the metatarsus (Taylor & Moore, 1958). These authors noted that in adult hens, medullary bone only developed in those bones of the pullet which contained haemopoietic tissue and therefore selected the tarsometatarsus as a bone which did not normally contain medullary bone. In laying birds, fracture resulted in deposition of medullary bone in the tarsometatarsus, though in smaller amounts than is seen in a bone which naturally contains medullary bone. This was accompanied by a weight gain in the tarsometatarsus and a highly significant weight loss in the tibiotarsus and femur of the same leg. Weight loss from the femur and tibiotarsus was shown to principally affect the cortical bone. Medullary bone was entirely absent from the non-fractured contralateral tarsometatarsus from the same bird. However, fractured tarsometatarsi from non-laying birds healed as rapidly as in laying-birds; although both medullary bone and fracture callus were both derived from the endosteum, oestrogen clearly influenced the type of bone produced. The authors concluded that blood supply was an important factor in determining whether a particular bone developed medullary bone, but that since fracture resulted in the deposition of smaller quantities than in bones in which it is normally present, other factors must also be involved.

Another factor which clearly influences the amount and distribution of medullary bone within a bone is pneumatisation. The penetration of diverticula of the air sacs into the avian skeleton was noted hundreds of years ago (King, 1957), and has been the subject of much work, with contradictory and confusing results. Much of this confusion arose from the comparison of different species, but a study of laying fowl (King, 1957) determined the extent of pneumatisation in the domestic fowl. The cervical vertebrae (except the atlas and axis) were pneumatised, as were the thoracic vertebrae (except the fifth), the lumbro-sacral mass, the pelvic girdle, the first two vertebral ribs, the plate and cranial processes of the sternum, the humerus and the distal half of the coracoid. There was however, an element of inter- and intra-individual variation noted; different sides of the body were often aerated to varying degrees.The walls of the air sacs consist mainly of a thin layer of simple squamous epithelium supported by a small amount of connective tissue (Salt & Zeuthen, 1960). The cervical sacs are small paired sacs below the vertebral column whose diverticula pneumatise anteriorly, the

cervical vertebrae and posteriorly, the thoracic vertebrae. The interclavicular air sac has three separate pairs of diverticula which pneumatise the humerus, scapula and ribs. Two diverticula are derived from the largest air sac, the abdominal air sac; one of these pneumatises the synsacrum and pelvis, the other surrounds the head of the femur (Salt & Zeuthen, 1960).

In the present experiment, one of the ten 48 week-old hens examined had humeri which were not pneumatised. These humeri contained medullary bone of the quantity and distribution normally seen in the tarsometatarsus. The humeri of the remaining birds were pneumatised to varying degrees. This does not agree with King (1957), who concluded that the humerus of the domestic fowl, together with the cervical and thoracic vertebrae, are bones which are probably regularly completely aerated. In those humeri which were fully pneumatised, the air sac epithelium appeared to affect the development of the medullary bone, which formed in resorption cavities within the cancellous bone trabeculae or cortices. Similar observations were made on the free thoracic vertebrae of both the 48 week-old birds and those commercial layers sampled at 30, 50 and 70 weeks of age. This bone was pneumatised in all the birds sampled, and appeared to be the only thoracic vertebra which was pneumatised, contrary to the findings of King (1957).In the free thoracic vertebrae, the erosion of cancellous and cortical bone and its replacement with small spicules or linings of medullary bone was particularly marked, especially in 50 and 70 week-old hens. The free (or 6th) thoracic vertebra is commonly associated with fractures in the domestic fowl (Riddel et al, 1968; 1969), leading to cage layer fatigue or paralysis. It is the only freely movable vertebra in the trunk and is subjected to considerable loading. Diminution of its structural bone is therefore likely to have serious consequences. Other bones which have been shown to commonly fracture in domestic fowl are the keel, humerus,and pelvis (Gregory & Wilkins, 1992). Although all of these bones have been shown to be pneumatised in domestic fowl and possibly structurally compromised by the formation of medullary bone, their fracture incidence is likely also to be related to their probable increased exposure to trauma. It can be seen however, that pneumatisation of a bone does not prevent medullary bone formation but does affect its distribution and location.

In this study, medullary bone was almost absent from those 70 week-old birds in which there was no follicular activity, and present in very small amounts in a number of others. In the birds which had ceased egg laying, there was evidence of renewed cancellous and cortical bone formation. Often remnants of medullary bone were sandwiched between cancellous or cortical bone lamellae.The effects of this sandwiching of woven bone with lamellar bone on the mechanical integrity of the skeleton are unknown but may possibly cause weakening. Previous studies have shown that medullary bone undergoes a regressive stage (Innoue, 1966) when the hen is more than 200 days old. During this stage, the network of medullary bone trabeculae disappears, becoming thin and fragmented. Later studies have shown that medullary bone is rapidly resorbed when egg-laying is stopped by a mineral deficient diet (Wilson & Duff, 1991). It has also been shown that there is renewed cancellous bone formation in birds which have naturally ceased egg production and which no longer exhibit any follicular activity (Wilson et al, 1992).

Medullary bone is clearly present in the modern domestic fowl for long periods of time, during which it is subjected to a relentless cycle of formation and resorption to meet the demands of egg production. In the study performed by Innoue (1966), no birds had medullary bone before 25 weeks of age and all birds aged 70 weeks were considered to be in a state of medullary bone regression. In this study, the hens came into production around 18 weeks of age and the majority of 70 week old hens sampled were still in lay. Egg-production has increased by approximately 25% since 1966 (Paice, 1993). Medullary bone is present for most of the commercial modern hen's life and plays a crucial role in its calcium metabolism (Miller, 1992). This function of medullary bone becomes more important with each improvement in production.

The morphology of medullary bone

Just prior to the onset of egg-laying, fine bony spicules develop from the endosteal wall of the cortex, becoming a dense meshwork of medullary bone which fills the outer third or half of the marrow cavity by the time the bird lays a single egg (Bloom et al, 1958). Medullary

bone stains blue with hamatoxylin and eosin, compared with pink-staining of cortical and cancellous bone. These staining differences are due to differences in the non-collagenous matrix component, the collagen and mineral components being similar to cancellous and cortical bone (Dallemagne, 1948;Taylor & Moore, 1956; Simkiss & Tyler, 1959; Stringer & Taylor, 1961; Stringer, 1962; Taylor et al, 1971).

Enormous inter-individual variation in the amount of medullary bone has been shown to occur (Riddell et al 1969; Wilson & Duff 1990). In the femur, for example, which probably has a greater proportion of medullary bone than other bones, medullary bone can occur as a massive network almost filling the marrow cavity, or as scattered spicules. Riddel et al (1969) described a ratio of 74:0:26 medullary bone:osteoid:marrow in an internal layer. In undecalcified sections, it can be seen that medullary bone is normally well mineralised, with fine osteoid borders only visible at high magnifications (Bloom et al 1958; Riddell et al, 1969; Wilson & Duff, 1990, 1991).However calcium, phosphorus, or vitamin D_3 deficiency causes an osteomalacia of medullary bone in laying hens in which the proportion of osteoid is increased dramatically (Doyle 1925; Bloom et al 1958; Simpson et al 1964; Riddell et al 1969; Wilson & Duff, 1991). All the hens sampled in this study had varying quantities of medullary bone which was well mineralised.

The cells associated with medullary bone in the laying hen have been described in detail (Bloom et al, 1958; Innoue 1969; Riddell et al 1969). Generally it is agreed that spicules of medullary bone are associated with abundant osteoblasts which were either spindle-shaped, or at right angles to the bone surface with plump nuclei. Medullary bone trabeculae contained scattered osteocytes and many osteoclasts are also apparent, with multiple nuclei and dark-staining cytoplasm. The medullary bone examined in this study was associated with similar cells and it was noted that no flattened bone lining cells of the type seen on the surface of cancellous bone were seen. There is no description of such a cell type in medullary bone and this may be representative of its rate of bone turnover.

In conclusion, medullary bone in the modern laying hen differs in its distribution but not

morphology from that of its counterpart of 30 years ago. Medullary bone is present in more bones and for a longer period of time. The distribution and location of medullary bone in pneumatised bones suggests a possible role in the diminution of structural bone. The absence of cancellous bone osteoid in laying birds, and evidence of renewed cancellous bone formation in birds which have ceased egg production also indicate that medullary bone is associated with changes in cancellous bone formation.

Osteoporosis

The term osteoporosis refers to a group of conditions in which the mass and structure of the skeleton are altered in a way which increases fracture risk. It is a common condition in the ageing human population, where it most frequently affects post-menopausal women (Lindsay & Cosman, 1992). Fractures of the spine, femoral neck, and distal radius are most common, and are preceded by a prolonged period of asymptomatic bone loss. The risk of fracture is proportional to the amount of bone present per unit volume of the skeleton because mass gives bone 80% or more of its strength (Johnson 1989).

Although metabolic disorders which result in bone loss can be detected by a variety of methods (radiographic, biochemical etc.), bone biopsy is required for a precise diagnosis of osteoporosis. Plastic embedding methods in conjunction with double tetracycline labelling allow differentiation between mineralised bone and osteoid as well as dynamic measurement of bone formation. Histomorphometric assessments of bone samples distinguish between osteoporosis and subtle osteomalacia, as well as yielding valuable information on bone turnover and trabecular architecture. Histomorphometric analysis of bone from post-menopausal osteoporotic bone reveals sub-normal cancellous bone area, usually accompanied by increased marrow fat (Meunier et al, 1971). Trabecular microstructure is also affected; there are significantly fewer, less well connected trabeculae in trans-ileal specimens from individuals with vertebral fractures (Kleerekoper et al, 1985). Although cortical width is often decreased, with accompanying cancellisation of the endocortical border, cortical porosity is usually normal (Brown et al, 1987). In 70% of cases, bone formation rates are low (Carasco et al, 1989; Marie et al ,1989), but high-turnover osteoporosis can also occur (Brown et al, 1987; Eastell et al ,1988).

Numerous risk factors for osteoporosis have been identified; e.g. race, sex, familial prevalence, low calcium intake, high alcohol intake, smoking, and low physical activity levels (Lindsay & Cosman, 1992). However, the single most important factor to influence the rate of bone loss is the cessation of ovarian function, regardless of age at which it occurs (Aitken et al, 1973; Richelson et al, 1984). The cancellous bone of the vertebral bodies is

most severely affected by the loss of oestrogen (Johnstone et al, 1984). The pre-menopausal decline in ovarian activity has also been demonstrated to result in bone loss, but at a lesser rate (Riggs et al, 1982).

In young adults, it is thought that each remodelling unit results in exactly the same amount of bone being replaced by the osteoblasts as is removed by the osteoclast. However, with advancing age, an imbalance occurs in favour of the osteoclasts so that more bone is resorbed than the osteoblasts form (Parfitt,1981;1984). Therafter, the activation frequency of remodelling cycles determines the overall rate of bone loss (Steiniche et al, 1989). Oestrogen deficiency appears to both increase the activation frequency of remodelling units, and to increase the deficit which occurs on completion of each remodelling cycle (Eriksen et al, 1984; Parfitt, 1988; Steiniche et al, 1989).

Bone mass at any stage in an individual's life is determined by the mass achieved during growth and subsequent consolidation minus any losses which occur as a result of remodelling. It would clearly be advantageous to maximise peak bone mass in order to offset subsequent losses. Peak bone mass is principally controlled by genetic factors (Smith et al, 1973; Maller et al, 1978; Pocock et al, 1987; Dequeker et al, 1987), but may be positively influenced by increased calcium intake prepubertally (Matkovic et al, 1988). During the period of consolidation (early adulthood), peak bone mass may also be increased by increased activity and calcium intake (Kanders et al, 1988).

Bone Fragility in Laying Hens

Bone fragility in productive layers was first reported by Couch (1955) , who termed the condition "cage layer fatigue". He described a condition in hens that had a high (70-85%) rate of production coupled with an excellent feed efficiency. Affected birds "came down with some sort of leg trouble" and had bones "so brittle that the ribs gave way, causing the heart to be punctured". Couch concluded that the condition might be related to nutritional deficiency but the problem did not respond to treatment with vitamins, calcium, or phosphate.

Similar reports of the condition were made by other authors (Francis,1957; Grumbles,1959; Snoeyenbos et al, 1957)

The first detailed description of the pathology of cage layer fatigue was that of Bell & Siller (1962). They studied 40 cases of cage layer fatigue which occurred in the course of a wider breeding experiment involving 624 birds. These cases were confined to those birds which were caged and selected for either intensive laying or large eggs. The condition was reported to occur in two forms; in the peracute form, death occurred suddenly in apparently healthy birds, while in the acute form birds were unable to stand immediately after oviposition. Some such birds died during this stage but those which survived a few days made a full recovery to normal laying after hand-feeding. The condition only affected birds undergoing their initial heavy laying period. Radiographs showed cortical thinning of the long bones of both acute and peracute cases, but recent fractures were rarely observed. However, histology demonstrated abnormally thin cortical bone, containing numerous resorption cavities. Cancellous bone trabeculae had virtually dissapeared and osteoclasts had increased in number and size. There were also marked signs of increased formation; osteoblasts were cuboidal and closely packed on the medullary bone surface. Although very fine borders of osteoid were occasionally visible on the residual medullary bone trabeculae, osteoid was not generally present. Plasma total calcium, magnesium, and inorganic phosphate were normal in affected birds, but plasma alkaline phosphatase was increased and acid phosphatase levels decreased. Femur and tibia ashing results were similar to normal birds, indicating normal mineralisation, but breaking strength was reduced.

The authors concluded that a genetical factor played an aetiological role in the disease, which they more accurately described as osteoporosis. Caging was also implicated, since none of the affected bird's litter-housed full sisters developed the disease. They ruled out the involvement of dietary calcium or vitamin D deficiency because plasma calcium levels were normal as were rate of lay and shell quality, and osteomalacia was not observed.

Outbreaks of cage layer fatigue, or osteoporosis, continued to be reported throughout the

1960's in the U.S., the U.K., Australia, and Germany (Harms 1962; Gardiner,1964;Smetana, 1965; Gibson, 1966; Hartwigk, 1966; Scheifer & Dorn, 1969). Although there were few severe cases described throughout the 1970's, the bone fragility problem persisted (Bastien et al, 1979; Riddell, 1981). Gregory & Wilkins (1989) found that 29% of end of lay battery hens had sustained fractures by the time they reached the waterbath stunner at the processing plant, and 24% after depopulation from the battery (Gregory & Wilkins, 1992). This latter figure was almost halved by handling the birds individually rather than in groups with each bird held by one leg. Much of the damage occurred in the ventro-caudal protruberance of the keel and the rear margin of the ischium, both areas most likely to suffer trauma on withdrawal from the cage. In battery hens the proportion of fractures which had occurred before depopulation was 5% (the same percentage of fractures sustained by pigeons), while in perchery systems this figure was 25%. This increased fracture incidence is probably due to an increased likelihood of trauma in the perchery system, and is indicative of an underlying bone fragility problem in laying hens regardless of their housing system.

Randall & Duff (1988) reported an increasing incidence of layers in which bone disease had been recorded as a cause of death, at an incidence of approximately 5%. This incidence had shown a variable response to dietary supplementation. Avulsion of the patellar ligament together with part of its bony insertion was observed and radiographs demonstrated a diminished quantity of bone tissue which was described as osteopenia. The lesions were therefore considered to be pathological fractures. Histopathological studies revealed that the birds were all osteoporotic, having normally mineralised bone occurring in reduced amounts. One further bird was diagnosed additionally as being osteomalacic; its medullary bone trabeculae occurred in a normal volume but demonstrated defective mineralisation, being composed almost entirely of osteoid. This study served to clarify the nomenclature of the condition, which had previously been confusingly and not very accurately referred to as cage layer fatigue. A later study (Wilson & Duff, 1991) investigated the effects of vitamin and mineral deficiency on the bones of laying hens. This study concluded that both dietary calcium deficiency and vitamin D_3 deficiency resulted in osteomalacic medullary bone trabeculae similar to those observed in the cases reported by Randall & Duff (1988).

Although much individual variation in osteoid seam width was observed in controls and phosphorus-deficient birds, the seams were never of the depth observed in osteomalacic birds.

Osteopenia is now regarded as the most significant disease of the skeleton in adult laying hens and is characterised by fragile bones and fractures (Riddell, 1992). 15-30% of hen mortality in the U.S. has been reported to be related to osteopenia (Roland & Rao, 1992). Production efficiency is further decreased by the presence of bone splinters in deboned meat from affected carcasses (Wilson et al, 1990). In the UK, where the welfare of battery hens is a matter of public interest, bone fragility in hens has been identified as a priority for welfare research (Perrins, 1992).

Although much work has been carried out regarding the effects of nutrition, exercise, and housing on bone breaking strength and ash in laying hens (Wilson & Harner, 1988; Hughes & Appleby, 1989; Knowles & Broom, 1990; Norgaard-Nielsen, 1990; Wilson et al, 1990; Gregory & Wilkins, 1992; Rowland & Rao, 1992), very little work has been carried out using histomorphometry. The benefits of biopsy and histomorphometry in the diagnosis and treatment of bone disease in man have been discussed previously, and even simple bone volume measurements have the potential to yield valuable information when applied to bone material from laying hens. A previous histomorphometric study of bone samples collected at 25, 42 and 60 weeks of age from male and female laying strain birds housed in cages or floor pens provided information on the progressive nature of cancellous bone loss in females during reproductive activity (Wilson et al, 1992). At 25 weeks of age the females already had considerably lower bone volumes than the males, and this bone loss continued through to 60 weeks of age (Figure 10.). A significant bone loss also occurred in males, but only in older birds. This study also demonstrated that while the cancellous bone trabeculae of all male birds were composed of mineralised bone and osteoid seams, osteoid was never present in cancellous bone from laying females. Fine seams of medullary bone osteoid were observed in these birds, and cancellous bone osteoid was present in out of lay birds whose medullary bone had been completely resorbed. The absence of cancellous bone osteoid in laying females was also noted by Bell & Siller (1962).

Figure 10. Undecalcified Von kossa-stained sections of the fifth thoracic vertebra from 60 week old male (top) and female (bottom) laying strain fowl. The diminution of cancellous and cortical bone in the female is obvious.

The effects of exercise and housing methods during rearing on bone volume at point of lay have also been investigated (Whitehead & Wilson, 1992), and were found to have no significant effect on cancellous bone volume. Perch use has been shown to result in a significant increase in cancellous bone volume compared to controls at the end of lay, but all the birds were considered osteoporotic. It was therefore concluded that some other factor or factors had a greater role in the development of osteoporosis in laying hens (Wilson et al 1993; Hughes et al, 1993).

Aims of the experiment

Cancellous bone volume in laying hens has been shown to decline progressively from the start of the laying cycle, and appears to occur in the absence of cancellous bone formation, but in the presence of medullary bone formation (Wilson et al, 1992). Cortical bone is not labelled by fluorochromes during the laying cycle, but medullary bone is intensively labelled during this period (Roland, 1990; Wilson & Duff, 1990). It has also been shown that many layers are osteoporotic at the start of lay, and although exercise can result in small differences in cancellous bone volume at the end of lay it does not prevent osteoporosis (Whitehead & Wilson, 1992; Wilson et al 1993; Hughes et al, 1993).

The effects of medullary bone modelling and remodelling on the structural skeleton during the laying cycle have not been investigated. It has, however, been observed that very little intracortical remodelling occurs during this period in quail, chickens, and pigeons fed calcium sufficient diets (Miller, 1992). The general aim of this study is to investigate the effects of medullary bone modelling and remodelling on the development of osteoporosis in laying hens. More specifically, the effects of reproduction on the formation and resorption of cortical, cancellous and medullary bone will be examined using fluorochrome bone labels and histomorphometric techniques. It is intended that such a study will provide further information regarding the timing and causes of bone loss in laying hens and to indicate possible remedial actions.

Animals

64 female day-old chicks (Hisex, Ross Poultry) were housed in a brooder until 4 weeks of age then transferred to individual cages. They were fed standard layer rations (see appendix), *ad libitum*, and from 14 weeks of age checked daily for egg-production. Eight birds were sacrificed weekly from 16 to 20 weeks of age and the remaining hens at 36 weeks of age by intravenous overdose of sodium pentabarbitone (Euthatal, Rhone-Merieux Ltd). Intravenous fluorochrome labels were administered as follows; oxytetracycline (25mg/kg body weight) and fluorescein complexone (20mg/kg body weight), 72 and 96 hours before sacrifice, respectively. Blood samples were collected immediately before sacrifice. The reproductive tract was examined and the diameter of the largest ovarian follicle recorded. The animals were subsequently grouped according to follicle size: [NFD] - birds in which there was no follicular development (n=5); [<9mm] - birds in which the largest follicle was less than 9mm in diameter (n=13); [>9mm] - birds in which the largest follicle was greater than 9mm in diameter (n=15); [1 egg] - birds which had layed a single egg (n=16); [mid-lay] - birds of 36 weeks of age which had layed eggs for approximately 20 weeks (n=15).

Blood Chemistry

2mls blood were collected from the brachial vein into heparinised blood tubes (Teclab, Lanarkshire), samples were centrifuged, and plasma frozen for storage.

Total plasma calcium was measured using a Wako calcium C kit (Alpha Laboratories, Hampshire) modified for use with avian body fluids. Modification is necessary because of the high calcium content of plasma from laying fowl. Defrosted plasma samples were therefore diluted 1:1 with phosphate buffered saline (pH 7.4) and mixed thoroughly before proceeding in accordance with the kit directions. Absorbance determination was carried out using an automatic plate reader.

Oestradiol was measured using Pantex Kit No. 047 (Biogenesis Ltd.). This is a

radioimmunoassay kit developed for clinical use but was modified for use on avian body fluids as follows:

Extraction

1. 10 ml E_2 recovery label and 0.3ml plasma were added to each extraction tube, vortexed, and incubated at room temperature for 15 minutes.
2. 6ml extraction mixture were added, shaken for 1 minute, then 5ml upper layer removed after separation and evaporated to dryness in a vacuum oven at 40-45^{o}C.
3. 800ml diluent was added, vortexed, and placed in a water bath at 37^{o}C for 30 minutes, and vortexed again.
4. 500ml of reconstituted solution was pipetted into clean glass tubes for assay

Assay

1. 100ml tracer and 100ml 1st antiserum was added to each tube, vortexed, incubated at room temperature for 20 minutes and centrifuged at 3500rpm for 10 minutes.
2. Supernatant was decanted and samples counted in gamma counter.

Dilutions for standards were adjusted accordingly.

Bone samples

The right proximal tarsometatarsus, tarsometatarsal diaphysis, distal humerus, humeral diaphysis, and femoral diaphysis were dissected from each bird, trimmed and processed as described in Chapter 1. for decalcified sections. The corresponding samples from the left appendicular skeleton were processed for undecalcified sections as described in Chapter 1. Three semi-serial 8mm undecalcified sections from each block were also cut and stained as described in Chapter 1. Extra 12mm sections were cut from each block and stored dry between filter papers. They were mounted in Fluoromount and immediately examined unstained under transmitted ultraviolet light with BG12 exciter filters and K530 barrier filter. Additionally, 1mm^3 pieces from the marrow cavity of the distal femur were processed for electron microscopy.

Ultrathin sections

$1mm^3$ samples for electron microscopy were fixed in 2.5% glutaraldehyde in 0.1M sodium cacodylate buffer for 1 hour at 4^oC. They were decalcified in 10% EDTA in 0.1M Tris buffer for 48 hours and post fixed in 1% osmium tetroxide in 0.1M sodium cacodylate buffer for 1 hour at 4^oC. Samples were then dehydrated in ascending concentrations of ethanol, transferred to inhibisol and embedded in araldite. Blocks were polymerised at 60^oC. Ultrathin sections were cut on a diamond knife, mounted on plastic coated copper grids (10% Formvar in chloroform) and stained with uranyl acetate and lead citrate. The sections were examined in a Philips EM 300 electron microscope.

Histomorphometry

Cancellous bone volume (CBV) and medullary bone volume (MBV) were calculated from toluidine blue-stained longitudinal sections of tarsometatarsus and humerus by point counting using an eye-piece graticule. Counts were made on three sections per block, over 12 fields per section, each field comprising 100 points (Figure 11.). Bone volumes were expressed as a percentage of the area measured. Cortical thickness was calculated from the mean of four measurements on toludine blue-stained diaphyseal sections, as shown in Figure 12.

Statistical Analysis

Results were analysed using the Macintosh version of Minitab (Release 6.2). For each parameter measured, group mean and standard deviation were calculated, and a Student t-test carried out to test significance. Cricketgraph (1.3) was used to display results.

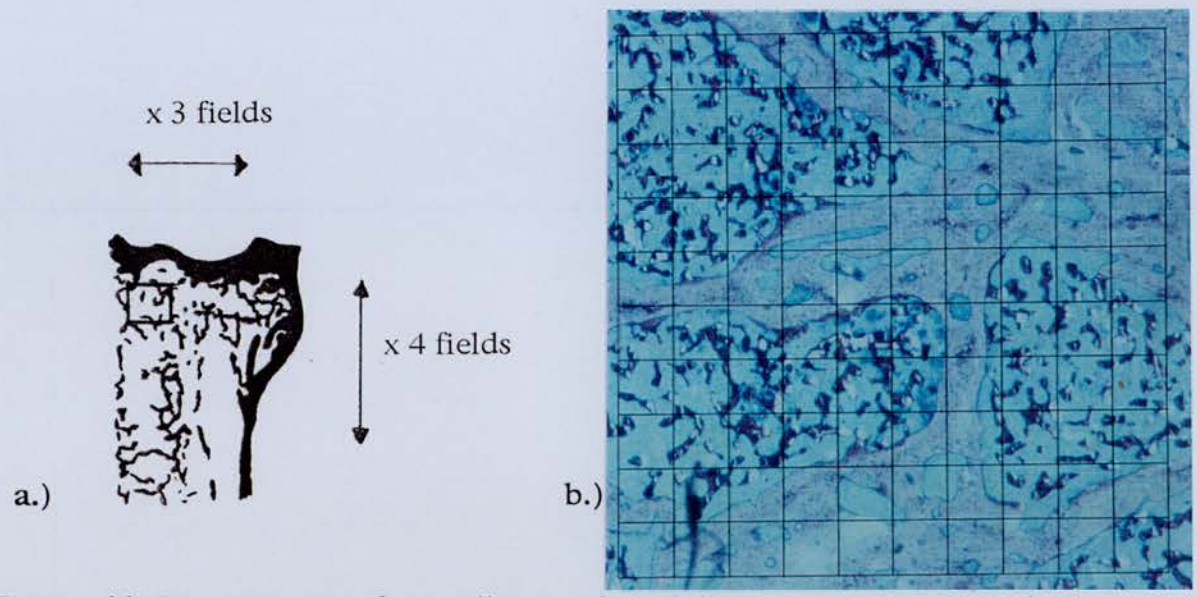


Figure 11. Measurement of cancellous and medullary bone volume in the proximal tarsometatarsus **a.)** number of fields measured on each of three undecalcified sections and **b.)** one field, comprising 100 counting points.

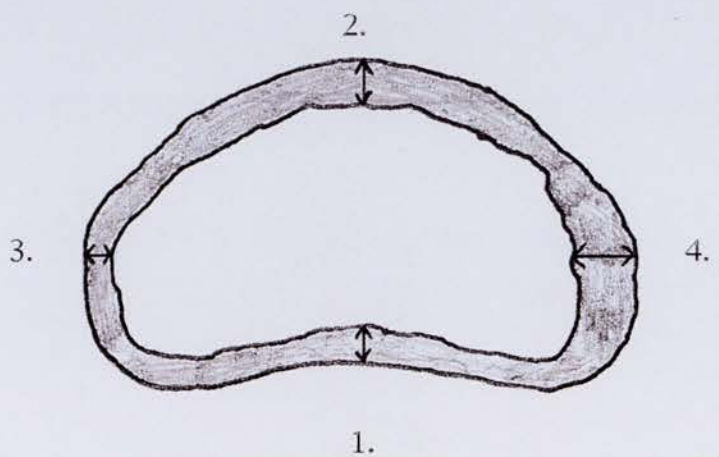


Figure 12. Measurement of cortical thickness in the diaphysis of the tarsometatarsus from the mean of four measurements on each section

Age

The ages of the birds in groups [NFD], [<9mm], [>9mm], [1 egg], and [mid-lay] are shown in Tables 1-5, respectively. Mean ages for each group are shown in Table 6. The difference in mean age between birds at the start of follicular development and birds which had layed a single egg was 14 days.

Reproductive tract

The diameter of the largest ovarian follicle of the birds in groups [<9mm] and [>9mm], are shown in Tables 2 and 3, respectively. Mean follicular diameter for each group is shown in Table 6. In birds in which lay had commenced, the diameter of the largest ovarian follicle was irrelevant and not therefore measured.

Blood Chemistry

Oestradiol and total calcium values for the birds in groups [NFD], [<9mm], [>9mm], [1egg], and [mid-lay] are shown in Tables 1-5, respectively. Mean values for each group are shown in Table 6, and displayed graphically in Figure 13.

Oestradiol values increased through follicular development, reaching a peak in the [>9mm] group before settling at a significantly higher level in the [1egg] and [mid-lay] groups than in the [NFD] group ($p< 0.001$).

Total calcium increased significantly ($p<0.001$) through follicular development to the [1egg] and [mid-lay] groups.

Histology

[NFD] group.

Examination of decalcified sections of proximal tarsometatarsus and tarsometatarsal diaphysis from birds which had no follicular development revealed the presence of thick cortices (Figure 15c), well connected, thick cancellous bone trabeculae and the complete absence of medullary bone (Figure 15c). Cancellous bone surfaces were covered principally

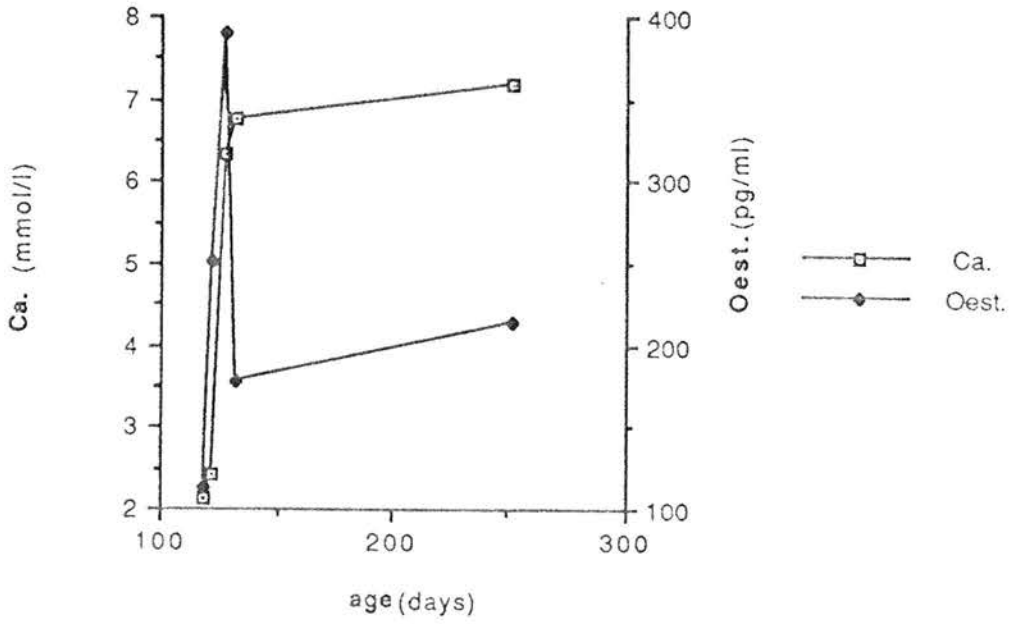


Figure 13 . Plasma total calcium (mmol/l) and oestradiol (pg/ml), from [NFD] through to 36 weeks of age [mid-lay].

by a flattened layer of bone lining cells, with occasional rows of rounded, active osteoblasts, and more rarely, single osteoclasts with up to 5 nuclei. The cancellous bone osteocytes had prominent nuclei and their lacunae were fairly large (Figure 14 a). The cortices showed no sign of remodelling, their Haversian systems being comprised primary osteones without cement lines.

The humerus of all the birds in the [NFD] group was pneumatised to varying degrees; in some, the air sac ended in the diaphysis while in others it extended throughout the bone. The air sac epithelium of the latter formed a tight lining to the cancellous bone trabeculae, usually with a flattened and elongated layer of bone lining cells between the two. The cancellous bone appeared plentiful, with a network of thick trabeculae extending through the bone.

Undecalcified stained sections showed the cortical and cancellous bone to be well mineralised, the latter having occasional osteoid seams. Under polarised light, the cancellous bone exhibited a typical lamellar structure. Unstained 12μm sections examined under transmitted fluorescent light demonstrated clearly the extensive presence of the two fluorescent bone labels (Figure 16a). These occurred as two narrow bands, each approximately 62μm wide, running parallel to most of the cancellous bone surfaces. The labels were approximately 100μm apart, the second label situated approximately 200μm beneath the bone surface. The cortices were unlabelled.

[<9mm] group

Decalcified sections of proximal tarsometatarsus and tarsometatarsal diaphysis in this group differed from the [NFD] group in that there were increased numbers of osteoclasts and the bone lining cells appeared plumper (Figure 14b). These activated bone lining cells were present on most cancellous and endocortical bone surfaces, giving them a distinctive beaded appearance . There was no medullary bone development.

All of the humeri were pneumatised to varying degrees. In those which were partially pneumatised, the distal extremity was similar in appearance to the proximal tarsometatarsus. The activated bone lining cells in the remainder appeared to push the air sac epithelium away from the cancellous bone surface.

Undecalcified stained sections were similar to those in the [NFD] group; cancellous bone trabeculae were well mineralised with occasional osteoid seams.

Fluorochrome labels showed a similar distribution to those in the [NFD] group.

[>9mm] group

A marked proliferation and activation of bone lining cells was apparent in decalcified sections of proximal tarsometatarsus and tarsometatarsal diaphysis, the beaded appearance described in the [<9mm] group occurring extensively in this group also. Additionally, in the diaphysis, short rows of cuboidal osteoblasts often 3 cells deep could also be seen (Figure 14c), and in some places small spicules of medullary bone had been modelled on the cancellous bone surface. Large numbers of osteoclasts were present in these areas of medullary bone modelling. One humerus was not pneumatised. Deep layers of osteoblasts were present but there was no medullary bone formation.

Undecalcified stained sections of proximal tarsometatarsus and tarsometatarsal diaphysis revealed little osteoid present in cancellous bone trabeculae, but the medullary bone spicules had narrow fringes of unmineralised matrix. Unstained 12μm sections examined under transmitted ultraviolet light demonstrated that both bone labels were similar in appearance to those in the [NFD] and [<9mm] groups.

[1egg] group

Medullary bone was present throughout the metaphysis and the diaphysis in decalcified sections of proximal tarsometatarsus. It was confined to cancellous bone surfaces where it

formed small spicules (Figure 15 a). Cancellous bone trabeculae appeared less well connected and in some places thinner than in the [NFD] group. Osteoblasts were present around medullary bone spicules but there was no evidence of the extensive osteoblastic activity described in the [<9mm] and [>9mm] groups. There were numerous osteoclasts present in areas of medullary bone formation which were resorbing both medullary and cancellous bone (Figure 14d). Medullary bone osteocytes were very large in comparison to those of the cancellous bone.

The cortices appeared similar in thickness to the [NFD] group, but the endocortical surfaces were lined in places with medullary bone.

There were also medullary bone spicules in decalcified sections of humeri. In pneumatised bones these were either in resorption pits within cancellous bone, or formed between the cancellous bone and the the air sac epithelium. Three birds in this group did not have pneumatised humeri.

Considerable variation in fluorochrome labelling occurred within the same bone sample; in some areas of the diaphysis, both labels were present as diffuse clumps situated in medullary bone spicules (Figure 16c). In other areas of the diaphysis, the first label appeared in cancellous bone, as narrow concentrated bands parallel to the bone surface, while the second label occurred diffusely in medullary bone spicules extending outwards into the marrow cavity from the cancellous bone surface (Figure 16b). In other areas, both labels occurred as narrow bands in cancellous bone, similar to that described for the [NFD] , [<9mm], and [>9mm] groups (Figure16c).

[mid-lay] group

The most outstanding features of decalcified sections of proximal tarsometatarsus and tarsometatarsal diaphysis in this group were the quantity and distribution of medullary bone (Figure 15b). Medullary bone spicules were present throughout the entire marrow cavity, extending into the metaphysis, and separated by small areas of lipid-filled marrow. Numerous massive osteoclasts (the largest having 15 nuclei), were visible around the medullary bone spicules, and osteoblasts occurred in variously sized groups in the same areas. Medullary bone osteocytes were large with prominent nuclei.

Cancellous bone trabeculae were sparse and fragmented, and the osteocytes situated most deeply within them appeared shrunken.

The cortices showed widespread evidence of extensive trabecularisation; they were thinned and porous, and linings of medullary bone were visible within the resorption cavities (Figure 15d).

Decalcified sections of humerus showed similar changes to the tarsometatarsus, having large quantities of medullary bone. The distribution appeared, however, to be limited by the air sac epithelium. 2 birds had humeri which were not pneumatised, and these contained the extensive distribution of medullary bone observed in the tarsometatarsus.

Undecalcified stained sections of proximal tarsometatarsus and tarsometatarsal diaphysis demonstrated normally mineralised cancellous and medullary bone; there was a complete absence of osteoid on the cancellous bone trabeculae, while at high magnification fine osteoid seams were visible around the perimeter of medullary bone trabeculae. Examination of 12μm unstained sections under transmitted ultra violet light demonstrated the complete absence of either bone label in cancellous and cortical bone. However both labels were clearly visible as separate bands in medullary bone spicules (Figure 16d) and had a more concentrated appearance than the medullary bone labels described in the [1egg] group.

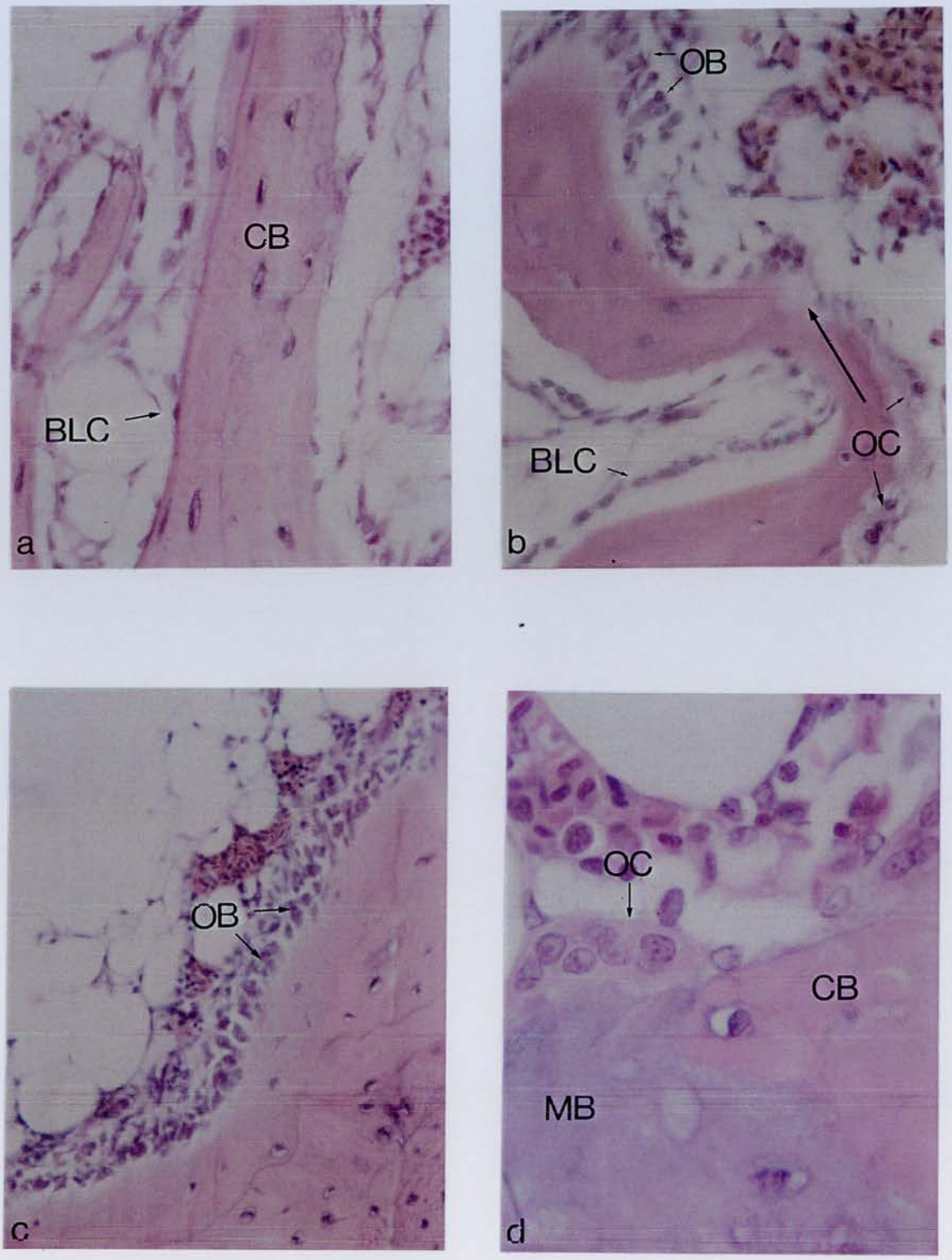


Figure 14. Decalcified sections stained H&E from a) [NFD] bird (x470); b) [<9mm] bird (x470); c) [>9mm] bird (x470); and d) [1egg] bird (x1500) CB-cancellous bone; MB-medullary bone; BLC- bone lining cell; OB- osteoblast; OC- osteoclast

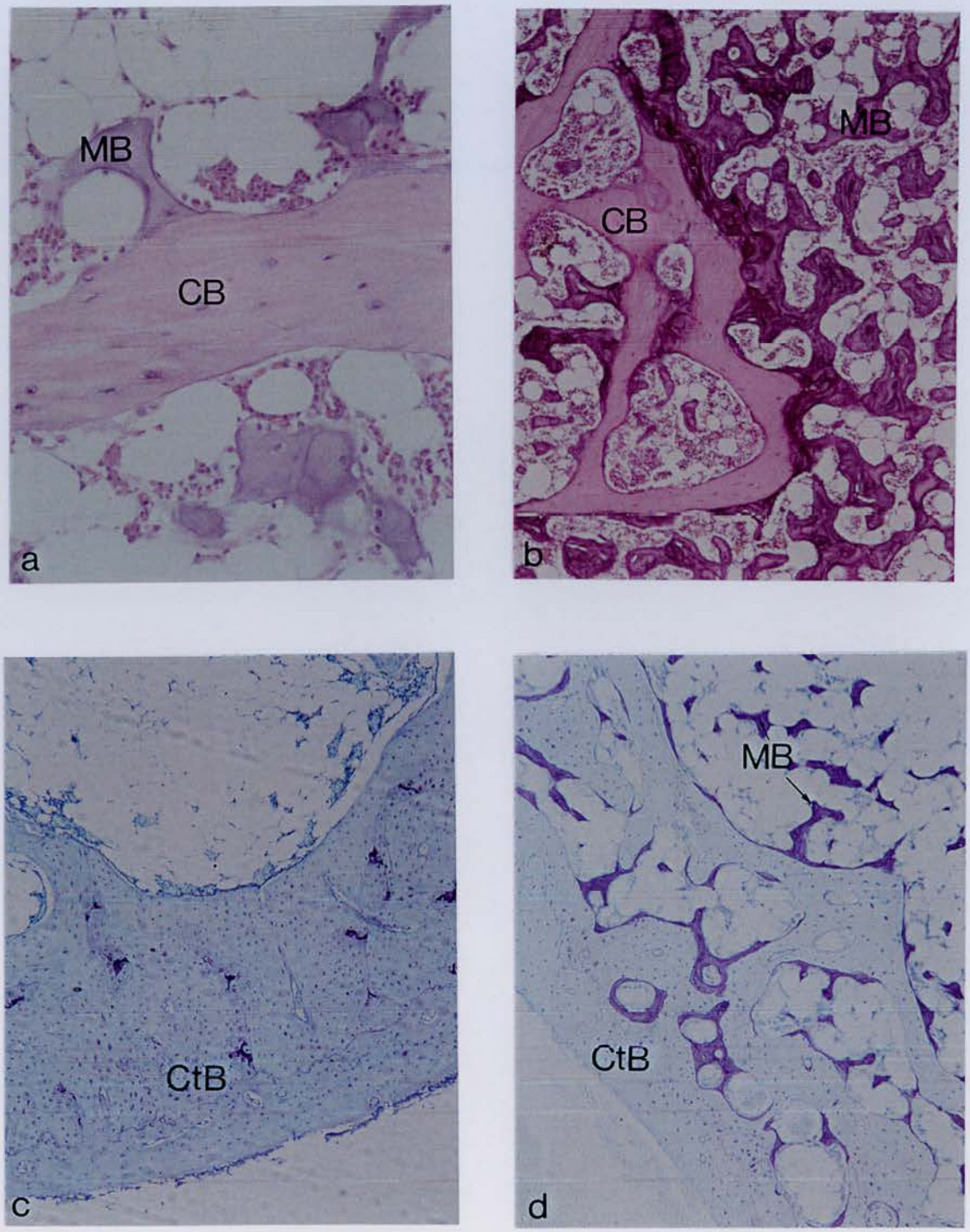


Figure 15. Decalcified sections of femur stained H&E from a) [1egg] bird (x240) and b) [ML] bird (x95)
Undecalcified tol. blue-stained sections of tarsometatarsal diaphysis from c)[NFD] bird (x95) and d) [ML] bird (x95)
CB- cancellous bone; MB-medullary bone; CtB- cortical bone

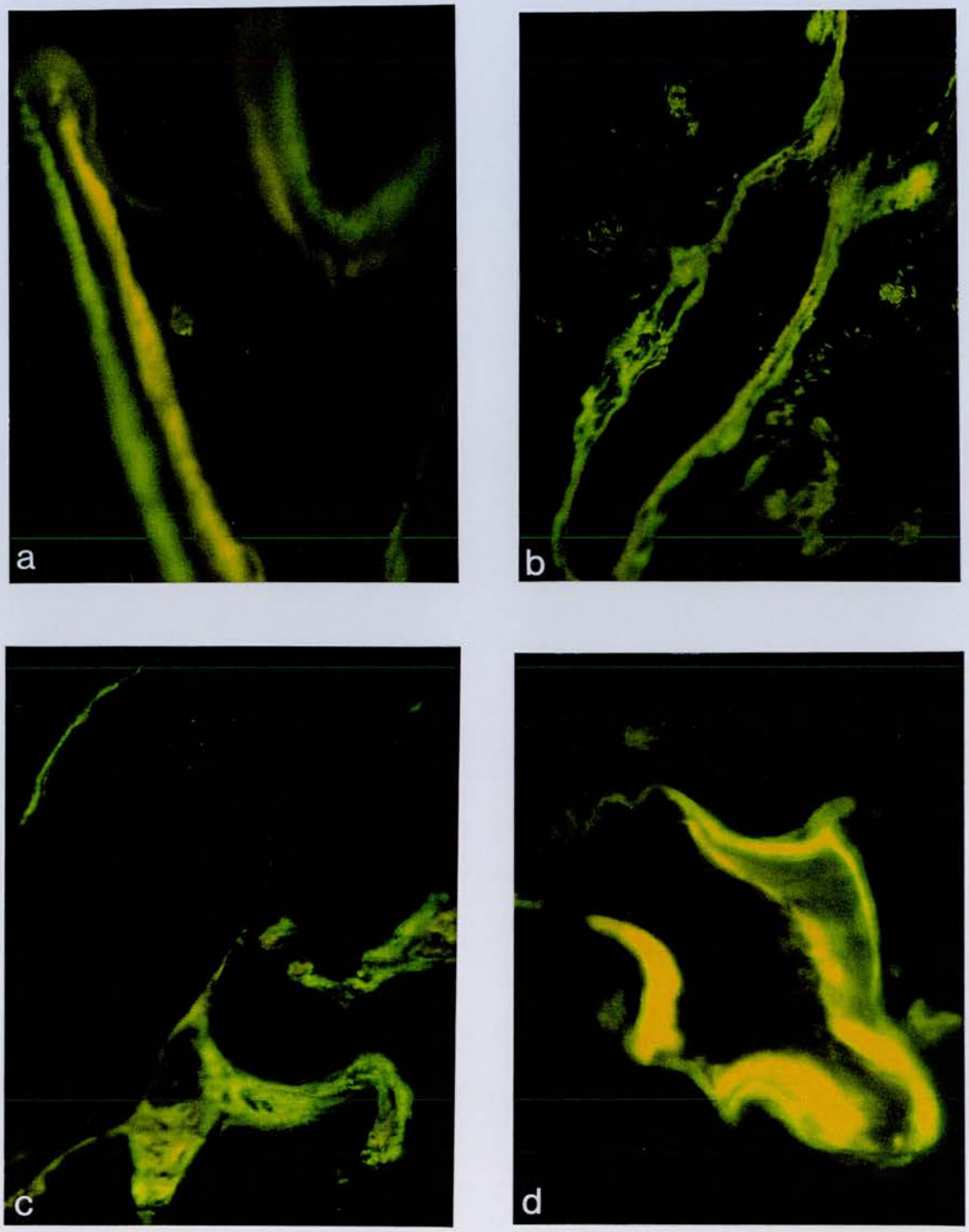


Figure 16. 12µm undecalcified, unstained sections labelled with oxytetracycline (OTC) and fluorescein complexone (FC). a) [NFD] bird (x470); b)[1egg] bird (x240) c) [1egg] bird (x470); d)[ML] bird (x470)

Ultrastructure

[NFD]

Most of the cancellous bone surfaces were covered by a layer of bone lining cells (Figure 17) which were situated approximately 0.15μm from the bone surface. They contained very little cytoplasm and their nuclei were flattened along the bone surface, measuring approximately 1x10μm. Occasionally active osteoblasts were observed in which the rough endoplasmic reticulum was dilated and the nucleus was placed away from the forming cancellous bone surface. No osteoclasts were seen. Osteocytes within the cancellous bone had a typical elongated appearance, with their long axis parallel to the collagen fibres.

[<9mm]

There were numerous osteoclasts observed in samples from these birds.The active osteoclasts were large cells with often 5 or 6 nuclei in section. They had well developed ruffled borders and the cytoplasm contained numerous mitochondria and lysosomal vesicles, giving it a foamy appearance (Figure 18). Some of the bone lining cells appeared to be activated, but most were similar to the [NFD] group.

[1egg]

Many active osteoblasts were visible adjacent to medullary bone spicules and were rounded in profile with dilated rough endoplasmic reticulum and eccentricaly positioned nuclei (Figure 19). These spicules were also associated with inactive osteoclasts, which were positioned close to the bone surface but did not have ruffled borders (Figure 20). Medullary bone osteocytes were all large and rounded, having more cytoplasm and retaining some rough endoplasmic reticulum and mitochondria. The osteocytes were often situated within irregularly shaped lacunae (Figure 21).

[mid-lay]

Cancellous bone osteocytes situated deep within the trabeculae often were shrunken in appearance, and the pericellular space filled with fibrillar material (Figure 22). Both active

osteoblasts and large osteoclasts were associated with medullary bone spicules , one osteoclast containing 23 nuclei in section. No bone lining cells were observed on medullary bone surfaces. Medullary bone osteocytes were all large and similar to those described in the [1egg] group.

Histomorphometry

Bone parameters for the birds in groups [NFD], [<9mm], [>9mm], [1 egg], and [mid-lay] are shown in Tables 1-5, respectively. Mean values for each group are shown in Table 6. Changes in bone parameters through follicular development and egg-laying are shown in Figures 23 and 24.

There was a significant decrease in proximal tarsometatarsal cancellous bone volume between the NFD group and the 1 egg group ($p< 0.001$), and although a further decrease occurred between the 1 egg group and the mid-lay group, the difference was not significant. Proximal tarsometatarsal medullary bone volume increased significantly ($p< 0.001$) from 0.00% to 2.67% between the NFD group and the 1 egg group, and increased further to 9.37% in the mid-lay group.

Cortical thickness of the proximal tarsometatarsus decreased insignificantly between the [NFD] and [1egg] groups, but significantly ($p<0.001$) between the [1egg] and [mid-lay] groups. Humerus cancellous bone volume decreased significantly ($p,<0.01$) between the [NFD] group and the [1egg] group and insignificantly between the [1egg} group and [mid-lay] groups. Medullary bone volume in the humerus increased significantly ($p<0.001$) from 0.00% to 6.89% between the [NFD] group and the [1egg] group.

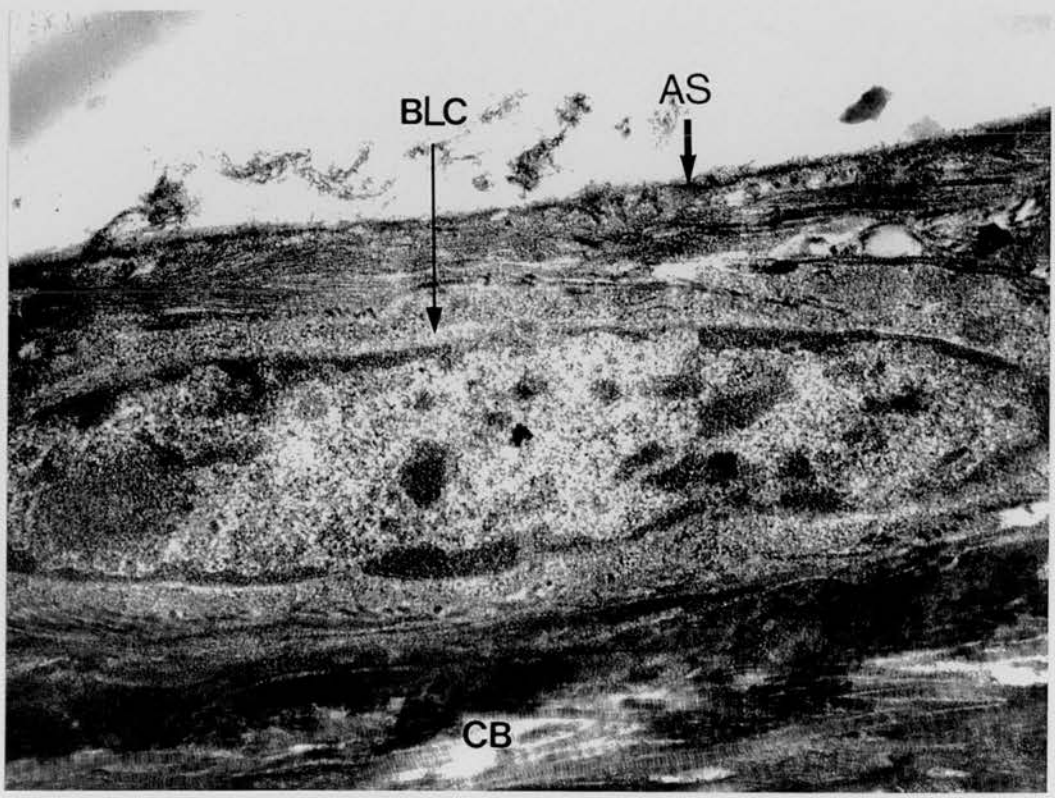


Figure 17. electron micrograph of humerus from [NFD] bird; bone lining cell (BLC) between air sac epithelium (AS) and cancellous bone (CB) surface (x29160)

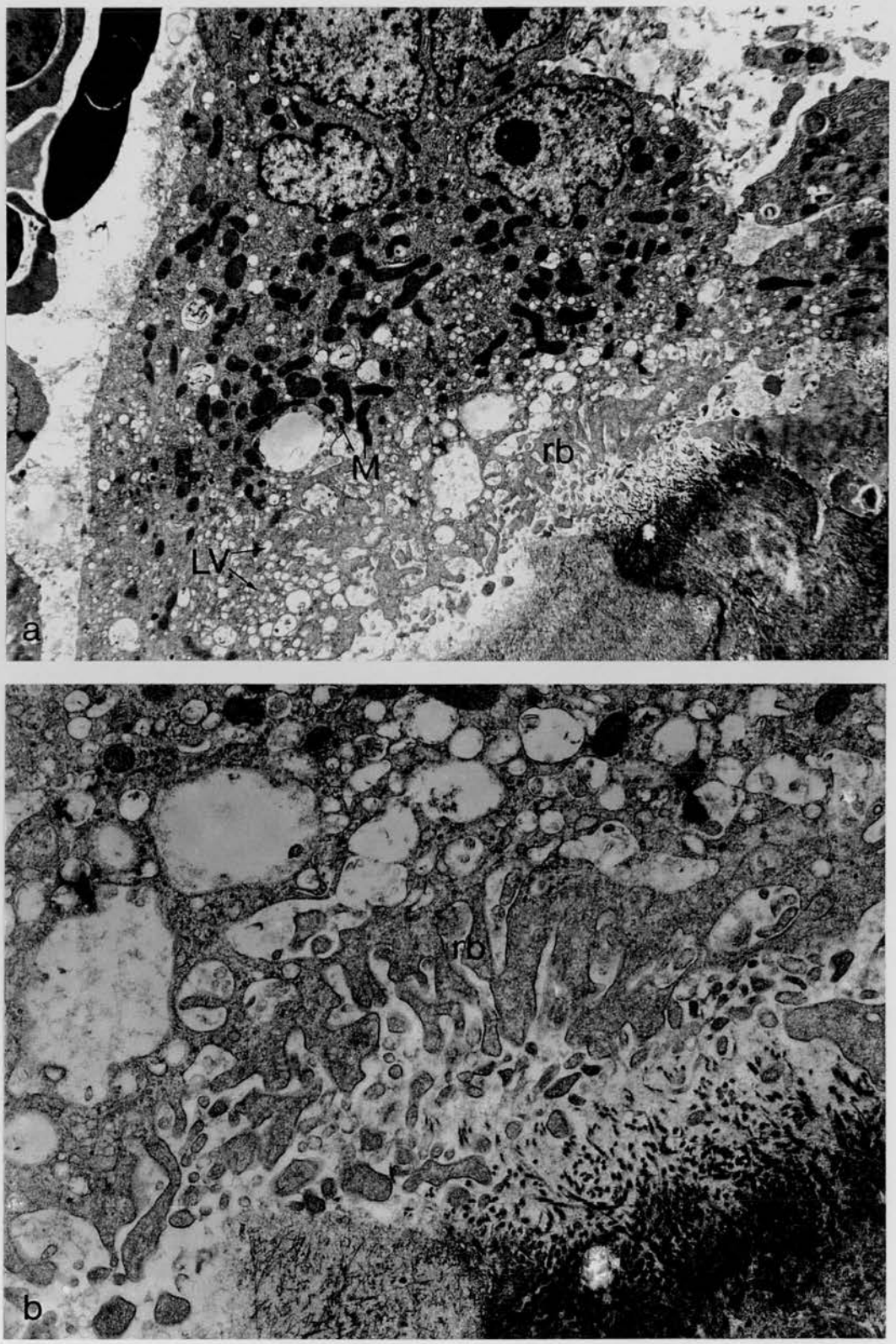


Figure 18. a) Active osteoclast from femur of [<9mm] bird (x4374) and b) detail of interface between ruffled border and bone surface (x16200). RB-ruffled border M-mitochondria LV-lysosomal vacuoles

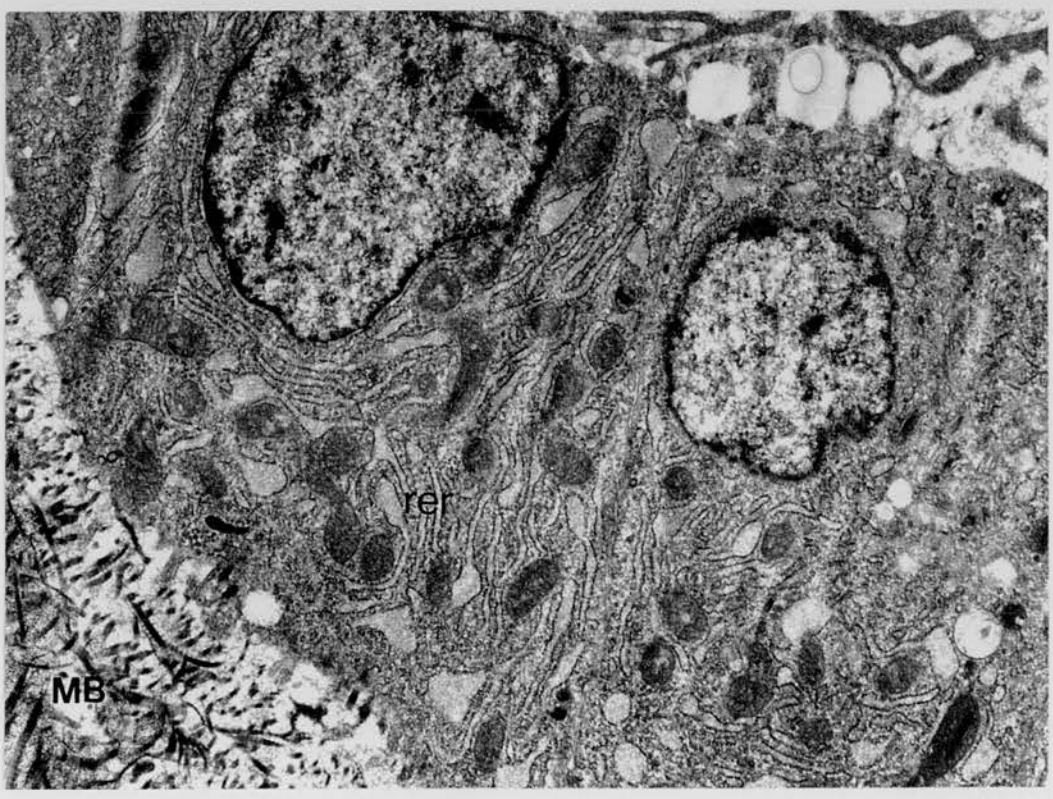


Figure 19. Active osteoblast from femur of [1egg] bird (x11664). MB-medullary bone RER-rough endoplasmic reticulum

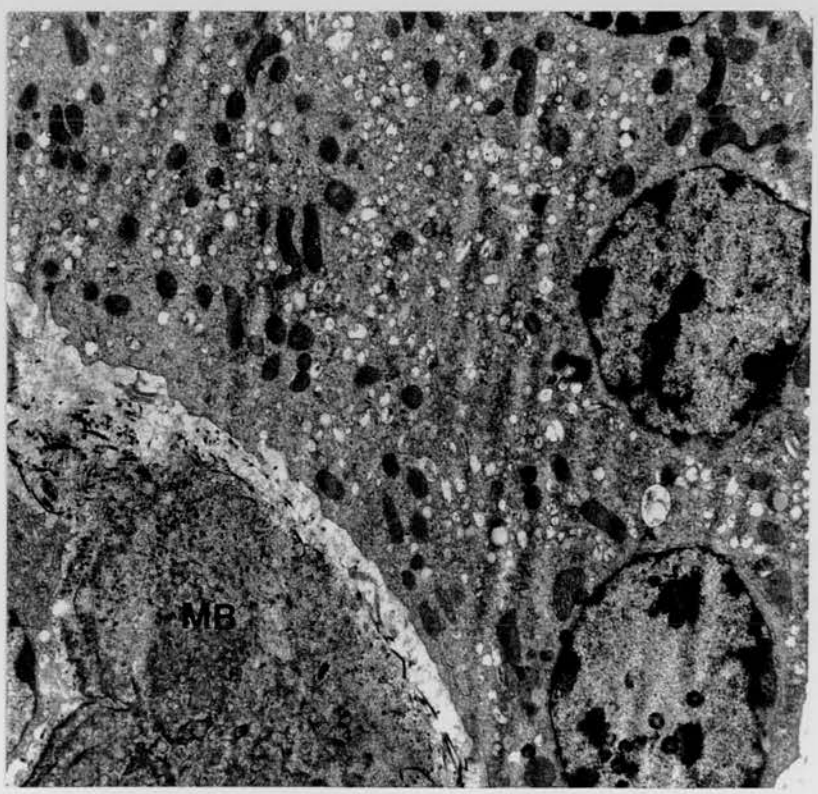


Figure 20. Inactive osteoclast from femur of [1egg] bird. MB- medullary bone (x7614)

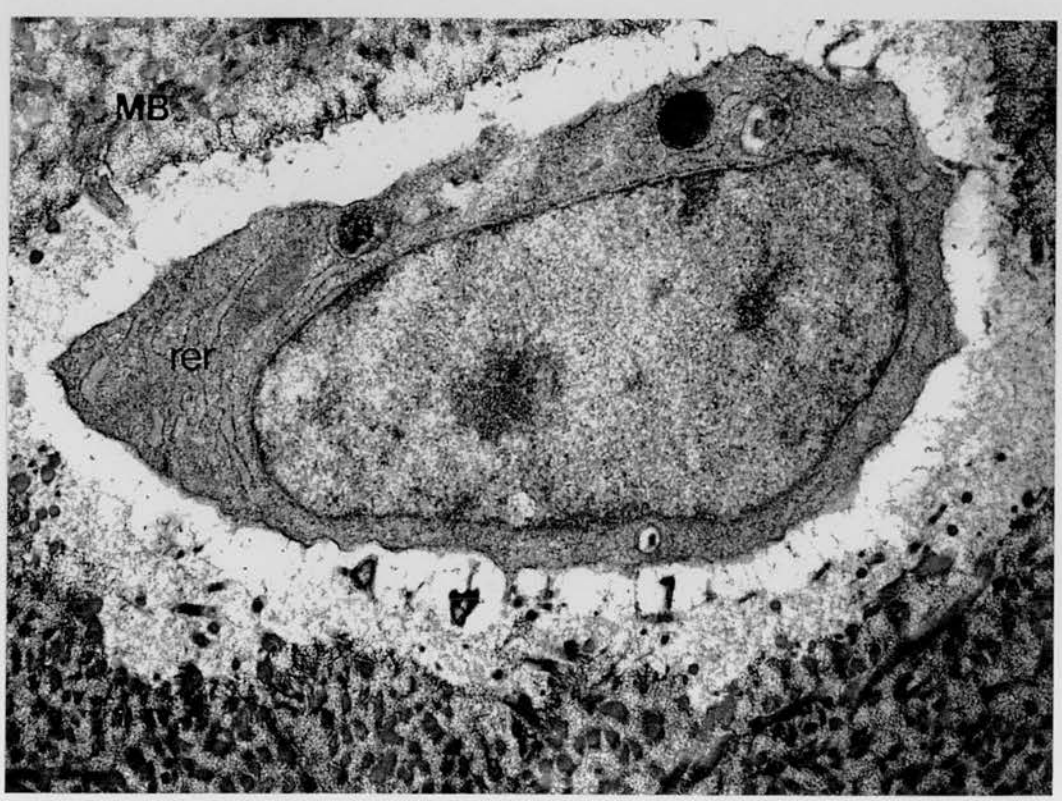


Figure 21. Medullary bone osteocyte from [ML] bird. RER- rough endoplasmic reticulum MB-medullary bone (x 18792)

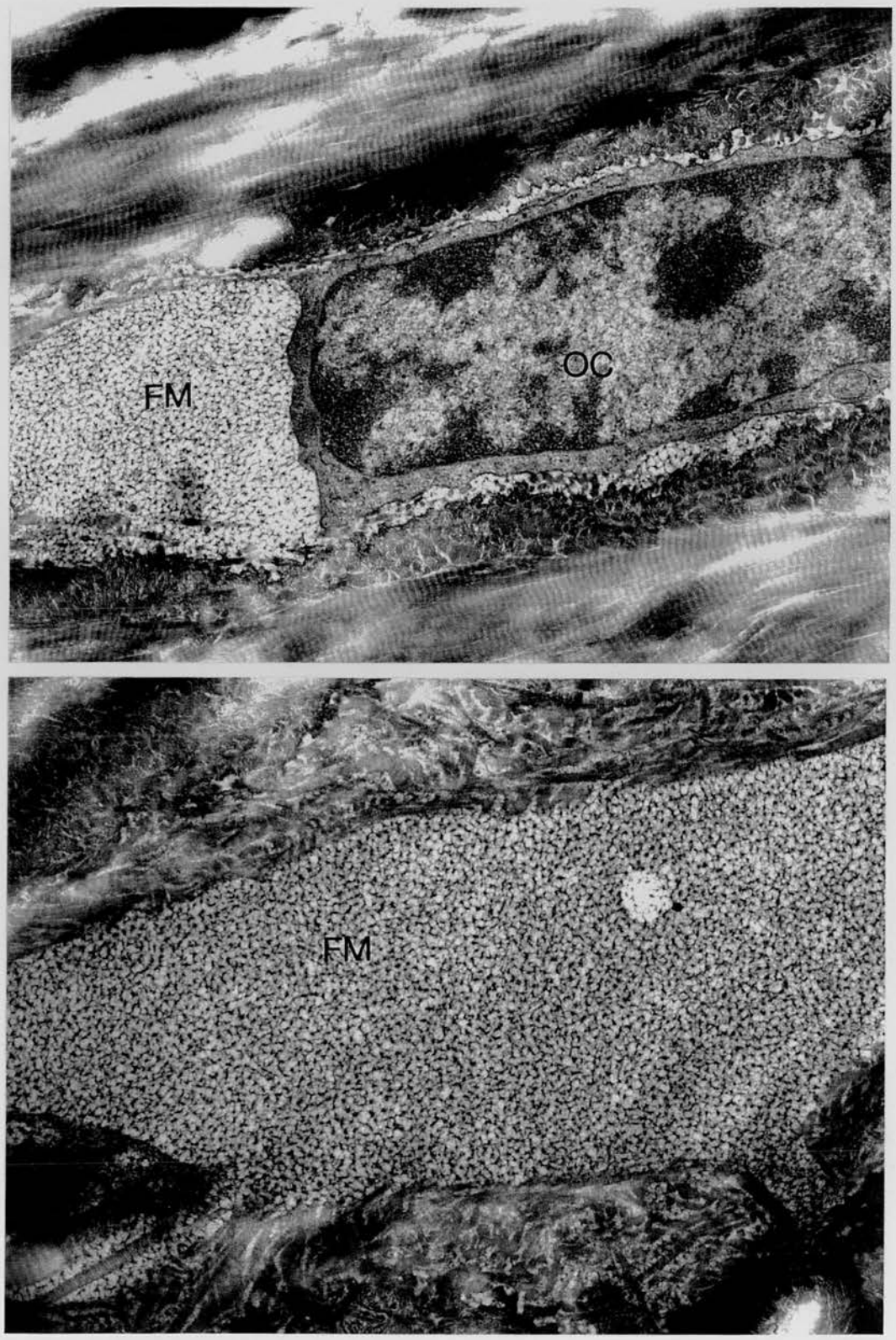


Figure 22. Cancellous bone osteocyte from femur of [ML] bird; a) x 18792 and b) x 23004. OC-osteocyte FM- fibrillar material

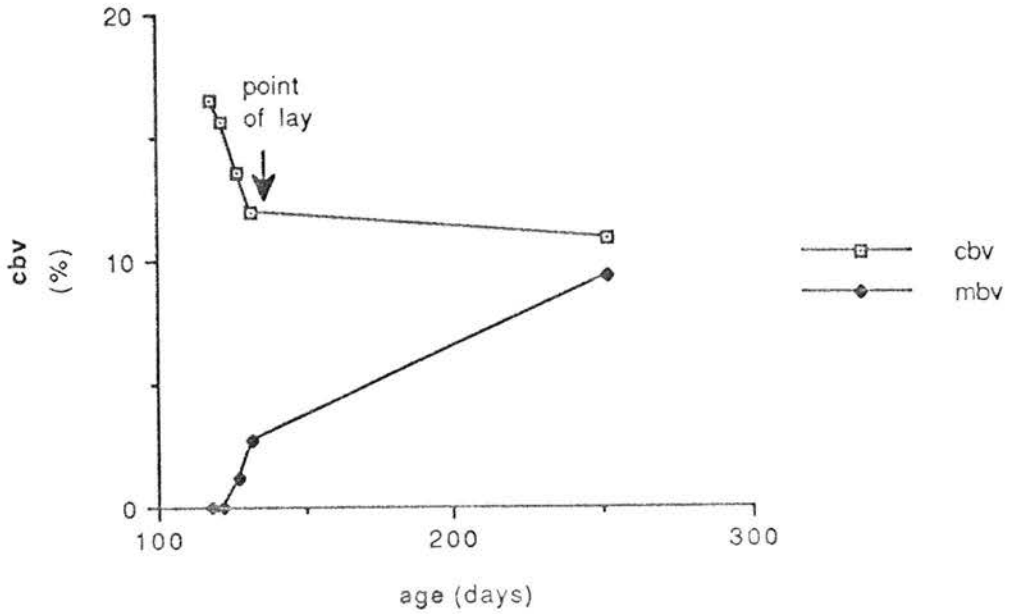


Figure 23 . Tarsometatarsal cancellous and medullary bone volumes, (%) from [NFD] through to 36 weeks of age [mid-lay].

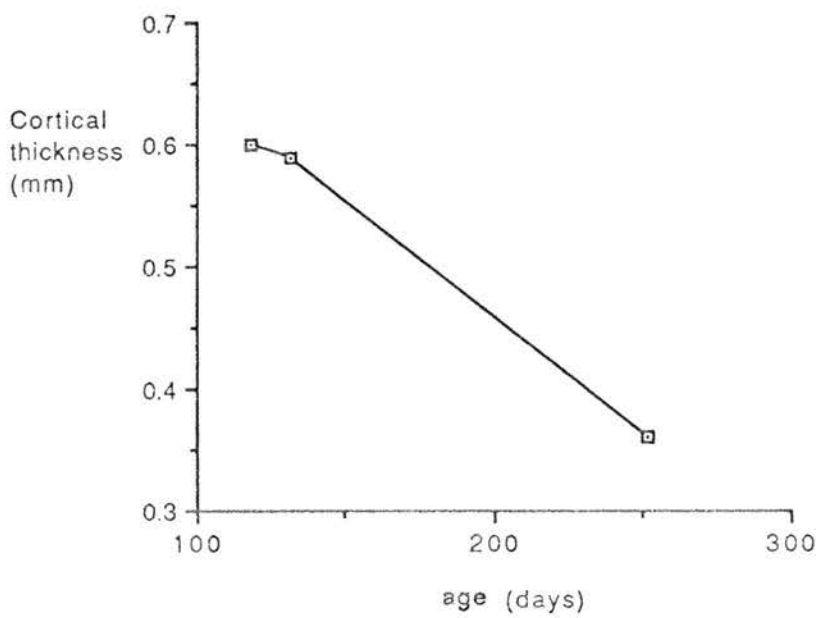


Figure 24 . Tarsometatarsal cortical thickness (%), from [NFD] through to 36 weeks of age [mid-lay].

Table 1. Age, plasma calcium & oestrogen, and bone parameters of birds with no follicular development (NFD)

bird number	age	follicle diameter	Total Calcium	Oestra-diol	PTM CBV	PTM MBV	PTM cortical thickness	Humerus CBV	Humerus MBV
	(days)	(mm)	(mmol/l)	(pg/ml)	(%)	(%)	(mm)	(%)	(%)
514	112	-	1.62	88.33	16.25	0.00	0.61	11.69	0.00
520	112	-	2.01	67.20	14.05	0.00	0.60	11.54	0.00
524	119	-	2.33	160.07	18.14	0.00	0.60	6.79	0.00
536	133	-	2.33	162.13	17.33	0.00	0.77	6.13	0.00
552	133	-	2.39	86.47	16.75	0.00	0.41	19.30	0.00

Table 2. Age, follicle diameter, plasma calcium & oestrogen, and PTM parameters of birds with follicles < 9mm in diameter.

bird number	age	follicle diameter	Total Calcium	Oestra-diol	PTM CBV	PTM MBV
	(days)	(mm)	(mmol/l)	(pg/ml)	(%)	(%)
510	112	5.5	1.33	239.97	16.69	0.00
511	112	3.6	1.33	244.33	13.19	0.00
512	112	4.5	1.25	146.20	14.00	0.00
513	112	3.7	2.25	344.07	*	*
515	112	6.3	1.53	279.20	*	*
516	112	3.0	2.23	241.02	17.29	0.00
517	112	3.9	1.25	225.37	20.61	0.00
519	119	5.8	5.06	231.07	*	*
525	119	5.4	2.69	259.00	15.54	0.00
528	126	8.2	5.29	368.33	12.22	0.00
530	126	7.5	1.93	255.83	*	*
548	133	6.5	3.18	215.53	16.55	0.00
550	133	5.0	2.21	224.70	14.42	0.00

* missing value

Table 3. Age, follicle diameter,plasma calcium & oestrogen, and bone parameters of birds with follicles > 9mm in diameter.

bird number	age	follicle diameter	Total Calcium	Oestra-diol	PTM CBV	PTM MBV
	(days)	(mm)	(mmol/l)	(pg/ml)	(%)	(%)
518	119	9.18	4.48	515.60	11.52	0.00
521	119	9.60	6.31	313.87	12.36	0.00
522	119	13.7o	4.72	243.40	11.66	0.03
523	119	10.90	3.77	424.47	14.67	0.00
526	126	10.60	5.46	412.83	15.83	0.00
527	126	9.40	7.62	326.93	16.80	0.00
531	126	23.60	7.54	377.37	11.89	0.00
533	126	24.90	6.48	915.10	13.80	1.64
538	133	24.60	8.86	469.37	13.50	4.92
542	133	20.40	7.38	322.17	13.53	0.94
543	133	28.00	5.12	244.27	13.08	1.43
546	133	13.60	5.68	359.40	12.33	4.28
549	133	27.40	8.53	192.27	15.08	1.75
554	133	26.90	7.90	226.53	13.25	1.25
557	133	9.60	5.07	501.97	14.94	1.46

Table 4. Age, plasma calcium & oestrogen, and bone parameters of birds which layed 1 egg.

bird number	age	follicle diam.	Total Calcium	Oestra-diol	PTM CBV	PTM MBV	PTM cortical width	Hum-erus CBV	Hum-erus MBV
	(days)	(mm)	mmol/l	(pg/ml)	(%)	(%)	(mm)	(%)	(%)
529	126	-	7.96	*	13.42	3.55	0.57	8.08	7.69
532	126	-	6.61	144.53	14.62	2.25	0.64	7.25	10.33
534	133	-	7.72	175.17	13.73	0.00	0.65	4.83	4.83
535	133	-	6.66	217.73	13.83	5.37	0.58	2.25	7.75
537	133	-	9.05	191.93	10.28	1.64	0.51	4.67	6.83
539	133	-	7.06	177.87	21.48	2.24	-	-	-
540	133	-	8.09	149.33	12.08	3.97	-	-	-
541	133	-	4.08	149.20	11.97	22.00	-	-	-
544	133	-	5.98	185.53	*	*	-	-	-
545	133	-	5.39	226.37	8.72	1.89	-	-	-
547	133	-	7.36	244.70	12.09	3.45	-	-	-
553	133	-	7.76	153.00	11.81	3.86	-	-	-
555	133	-	6.31	156.90	*	*	-	-	-
556	133	-	6.51	218.87	12.36	1.33	-	-	-
558	133	-	4.96	112.00	11.75	22.75	-	-	-
551	133	-	*	*	10.71	0.00	-	-	-

* missing value

Table 5. Age, plasma calcium & oestrogen, and bone parameters of birds aged 36 weeks (mid-lay).

bird number	age	follicle diam.	Total Calcium	Oestra-diol	PTM CBV	PTM MBV	PTM cortical width	Hum-erus CBV	Hum-erus MBV
	(days)	(mm)	mmol/l	(pg/ml)	(%)	(%)	(mm)	(%)	(%)
562	36	-	8.37	187.07	13.10	9.70	0.40	6.58	2.17
563	36	-	8.43	*	10.37	16.16	0.36	5.50	1.00
565	36	-	6.73	201.47	16.28	7.91	0.37	6.17	9.25
567	36	-	4.21	222.23	6.97	11.43	0.36	3.92	10.50
571	36	-	4.20	168.70	*	*	0.36	*	*
572	36	-	7.94	234.10	16.00	4.42	-	-	-
573	36	-	7.20	187.80	5.36	15.00	-	-	-
574	36	-	7.66	156.17	9.41	8.94	-	-	-
575	36	-	7.79	155.33	*	*	-	-	-
579	36	-	8.92	181.63	13.50	6.17	-	-	-
580	36	-	7.63	123.07	10.67	6.78	-	-	-
581	36	-	8.49	794.30	9.75	12.69	-	-	-
582	36	-	6.30	182.60	12.50	6.92	-	-	-
585	36	-	5.54	106.53	7.30	5.83	-	-	-
586	36	-	7.30	133.00	9.50	9.83	-	-	-

* missing value

Table 6. Mean Values (± S.D.)

	NFD	<9mm	>9mm	1 egg	36 weeks
Age days	121.80 (± 10.61)	118.46 (± 8.31)	127.4 (± 6.03)	132.07 (± 2.46)	252 (± 0.00)
Foll.Diam mm	-	5.3 (± 1.57)	17.49 (± 7.69)	-	-
PlasmaOest pg/ml	112.84 (± 44.83)	251.89 (± 55.94)	389.70 (± 176.48)	178.8 *** (± 37.7)	214.4 NS (± 173.6)
Plasma Ca mmol/l	2.14 (±0.32)	2.42 (± 1.36)	6.33 (± 1.57)	6.77 *** (± 1.31)	7.20 NS (± 1.40)
PTM CBV %	16.50 (± 1.54)	15.61 (± 2.53)	13.62 (± 1.58)	11.97 *** (± 1.60)	10.82 NS (± 3.34)
PTM MBV %	0.00	0.00	1.18 (± 1.56)	2.67 *** (± 1.44)	9.37 *** (± 3.60)
cortical thickness (mm)	0.60 (± 0.127)	-	-	0.59 NS (± 0.057)	0.37 *** (± 0.017)
Hum. CBV %	10.89 (± 3.97)	-	-	5.72 ** (± 1.91)	5.44 NS (± 1.66)
Hum.MBV %	0.00	-	-	6.89 *** (± 2.37)	5.99 NS (± 3.41)

NS	not significant
*	p< 0.05
**	p< 0.01
***	p< 0.001

The results from this experiment describe changes that occur in some bone and plasma parameters during the course of ovarian follicular development in laying strain fowl. Although the physiological changes which occur during this period are well documented (Lofts & Murton, 1973; Phillips et al, 1985; Johnson, 1986), and the process of medullary bone modelling previously described (Taylor & Stringer, 1965), their effects on structural bone were not known. The subsequent effects of medullary bone remodelling on structural bone volume had also not been previously investigated or quantified.

The reproductive system of birds consists usually of a single left ovary and its oviduct; the right ovary starts to regress around the tenth day of incubation. At hatch, the ovary of the domestic fowl contains millions of oocytes but only 200-500 of these will mature and ovulate. The oocytes become incorporated into ovarian follicles, which provide support during their growth and also cleave the yolk protein precursor vitellogenin, transported via the blood, into the two yolk proteins phosvitin and lipovitellin. A layer of granulosa cells forms around the oocyte, then a second thecal layer onto the granulosa layer and subsequently, further supportive layers cause the oocyte and follicle to extrude from the surface of the ovary. In the quiescent state, the ovary is compact, flat and triangular in shape, and the small follicles give it a granular appearance. Interstital cells within the connective tissue of the ovary produce steroids and during the onset of sexual development these, and the growing follicles secrete increasing amounts of oestrogens. These stimulate synthesis of vitellogenin in the liver and also growth of the follicles. As yolk deposition increases, the follicle becomes detached from the ovary but remains connected to it via the follicular stalk. The final stages of yolk deposition result in the rapid growth of the follicles from 8mm to 37mm in diameter in a 7-11 day period prior to ovulation. Follicles at various stages of development form a heirarchy, which gives the ovary a "bunch of grapes" appearance (Lofts & Murton, 1973; Phillips et al, 1985). During this time the thecal cells of the follicle secrete increasing amounts of oestrogen, but around 5 days before ovulation this decreases and the granulosa cell's progesterone production increases, promoting final development of the oviduct and appropriate changes in sexual behaviour.

In the present experiment, the difference in mean age between the birds in the [NFD] group and the [1egg] group was 10 days, and is consistent with the known timescale of follicular development (Lofts & Murton, 1973; Phillips et al, 1985). The plasma oestradiol measurements for each experimental group are appropriate for the observed follicular development; the [NFD] group had significantly lower oestradiol values than the other groups and the mean was similar to values recorded in other immature laying-strain fowl (P. Hocking, personal communication). Plasma oestradiol peaked in the [>9mm] group, and this is consistent with final phase of follicle growth in which the thecal cells maximally secrete oestrogen. The lower levels measured at later stages of the reproductive cycle are probably a consequence of the reduction in oestrogen secretion which begins approximately 5 days prior to the first ovulation. The birds in the [1egg] and [mid-lay] groups were at various stages in the egg-formation cycle when sacrificed. Plasma oestradiol levels fluctuate through the egg-formation cycle, from around 140pg/ml at the time of ovulation, rising to approximately 300pg/ml 6 hours before the next ovulation (Johnson & van Tienhoven, 1980). The results from the laying birds in this experiment fell into a similar range.

These results also serve to confirm that dividing the birds into groups according to the diameter of the largest developing follicle is a valid means of identifying their oestrogen status.

Measurement of total plasma calcium levels indicated that a significant elevation occurred in the [>9mm] group, and this increase was maintained through to the [mid-lay] group. Blood calcium circulates in two forms; as non-diffusable protein-bound calcium (unionized) and as diffusable ionized calcium. Nondiffusable calcium is bound by the plasma calcium binding proteins vitellogenin and albumin (Guyer et al, 1980). Oestrogen treatment increases total plasma calcium in avians, primarily by stimulating the production of blood calcium-binding proteins (Bacon et al, 1990) but also by increasing intestinal absorption of calcium via increased renal production of 1-α-hydroxylase (Castillo et al, 1977). This hypercalcaemic response to oestrogen is also exhibited in other sub-mammalian vertebrates (Dacke et al, 1973; Boelkins & Kenny, 1973). Thus, total plasma calcium increases in the period prior to

the commencement of lay, and this increase has been shown to be attributable to the protein bound fraction and not the ionized fraction. The magnitude of the increase in total plasma calcium when hens reach reproductive maturity has been reported by other authors to be 2.5-fold (Wideman, 1990), which is less than the 3.25 fold increase recorded in this experiment.

The histological observations reported in this experiment detail the changes which occur during reproductive development and the egg-laying cycle in bone of modern laying fowl. The process of medullary bone modelling has been extensively studied by oestrogen administration to male birds (Miller, 1977; Bowman & Miller, 1981, 1986; Oshashi et al, 1987, 1988, 1990, 1991) and the relationship between follicular development and medullary bone formation established in pigeons sixty years ago (Kyes & Potter, 1934). Further information is provided by histological studies of medullary bone formation (Bloom et al, 1941, 1942; Innoue, 1966) and of osteoporosis in the laying hen (Bell & Siller, 1962; Riddell, 1968; Randall & Duff, 1988) have been carried out. It has been observed that in periods of calcium deficiency, medullary bone remodelling is maintained at the expense of cortical bone (Taylor & Moore, 1954; Riddell, 1990), and that 50% of the phosphorus and at least part of the calcium from newly modelled medullary bone is derived from structural bone (Govaerts & Dallemagne, 1948; Hurwitz, 1964). However no previous attempt has been made to investigate and quantify the relationships between medullary bone modelling (through follicular development), medullary bone remodelling (through the egg-laying cycle) and histological changes in structural bone .

The histological observations made on samples from birds in the [NFD] group were consistent with a period of consolidation. This is a period during early adult life when bone mass continues to increase although linear growth has ceased, and is an important factor in determining peak bone mass (Lindsay & Cosman, 1992). The amount of bone accrued during this period is modifiable, and it has been shown that an increase in dietary calcium levels or physical activity can result in a 5% bone mass advantage in 30 year old women (Kanders et al, 1988). In this experiment, none of the birds retained a growth plate, and were therefore incapable of further linear growth. Fluorochrome bone labels administered 3 and 5 days

previously indicated extensive mineralisation of cancellous trabeculae, and the regular presence of unmineralised matrix seams was observed. This suggests that cancellous bone was being accrued.

The most important histological changes occurring between the [NFD] group and the [<9mm] group were the increase in osteoclast numbers in the latter, and also the apparent plumping of the bone lining cells. Activation of bone remodelling occurs on inactive bone surfaces, which are usually covered by flattened and extended bone lining cells. These cells may be involved in the propagation of the activation signal which initiates bone resorption (Miller at al, 1989). Bone lining cells are thought to be remnants of the team of osteoblasts that previously layed down bone matrix and are known to respond to bone resorption-inducing agents such as parathyroid hormone by contracting the boundaries of their cytoplasm, thus exposing the bone surface and allowing penetration by osteoclast precursors (Miller et al, 1976; Jones & Boyde, 1976). They also digest the thin layer of endosteal membrane which exists on quiescent surfaces and which is resistant to osteoclastic resorption (Chambers & Fuller, 1985; Delaisse et al, 1988).

The parathyroid glands of pullets enlarge during the pre-laying period (Macowan, 1931) and were considered by Taylor (1965) to have a possible role in stimulating calcium absorption. However, there is very little information available concerning circulating parathyroid hormone levels in avians because the lack of cross reactivity of the avian hormone with antibodies directed against mammalian parathyroid hormone means a suitable immunoassay is not available (McGregor et al, 1973). Although an HPLC technique for isolation and purification of avian parathyroid hormone has been described (Pines et al, 1984), no work to determine circulating levels in hens either during the period of ovarian follicular development or the laying cycle has been published. The stimulus for the observed activation of the bone lining cells and subsequent osteoclastic activity in this study remains a matter for speculation.

The cancellous and endocortical bone surfaces of the birds whose largest follicle was more than 9mm in diameter showed much osteoblastic recruitment and activity, resulting in the

deposition of the first medullary bone spicules. Most studies related to medullary bone formation describe the changes which occur on the endosteal surfaces of the cortices and indeed refer to the process as the "endosteal reaction" (Miller & Bowman, 1981; Schraer & Hunter, 1985; Oshashi et al 1987, 1988). Although the bone-lining cell changes observed in the present study did occur on endosteal surfaces, they were not restricted to this bone envelope and affected most cancellous bone surfaces also.

In the [>9mm] group, medullary bone was not labelled by flourochromes administered 3 days previously. The medullary bone spicules which extended from the cancellous bone trabeculae in this group were therefore formed within these three days, suggesting rapid bone formation and mineralisation. .

All of the birds in the [1egg] group had small spicules of medullary bone extending into the marrow cavity. This is entirely different to the appearance of pigeon bones at the onset of lay as described by both Kyes & Potter (1934), and Bloom et al (1941). Pigeon bones prior to the first egg being layed are described as having anastomosing medullary bone trabeculae extending in every direction throughout the marrow. Bloom et al (1958), reported that by the time the largest developing follicle had grown to 18mm in diameter a dense meshwork of medullary bone extended from the endosteal surface of the cortex through the outer half of the marrow. In the first detailed histological study of cage layer fatigue (Bell & Siller, 1962), the described amount and distribution of medullary bone in normal pullets was also quite different to that described in this study. Just prior to lay the medulla contained densely packed and closely knit medullary bone trabeculae, presenting only small intertrabecular spaces.

These differences may be a reflection of the enormous "improvements" in laying fowl brought about in the last 30 years. Given the observation that numerous osteoclasts were associated with both cancellous and medullary bone in point of lay birds, it is possible that a low medullary bone volume at the onset of egg-production is a disadvantage. Cancellous bone surfaces which had extensive coverage by medullary bone spicules would be physically protected , once egg-laying starts, from the cyclic, intensive periods of bone resorption which

occur during remodelling (van de Velde et al, 1985). However, such an increase in medullary bone volume before lay may conversely result in increased cancellous bone resorption during modelling.

The fluorochrome bone labels administered 3 and 5 days prior to sacrifice indicated that different sites in the same bone were involved in mineralisation of either cancellous bone alone, medullary bone alone or even a combination of the two. Where medullary bone was labelled, the fluorochrome was diffused throughout the whole spicule which often extended from the cancellous bone some 100μm into the marrow cavity. This clearly represents a massive apposition of new bone. Such a rate of bone formation could probably only be achieved with woven bone.

At mid-lay, medullary bone extended throughout the marrow cavity, and appeared similar in its distribution and quantity to that seen before lay in other studies (Bloom et al 1958; Bell & Siller, 1962). Fluorochrome bone labels were restricted to medullary bone, none being present in either cancellous or cortical bone, indicating no such bone formation. Medullary bone labelling was widespread throughout the bone, indicating massive bone turnover, but labelling within each medullary bone spicule was concentrated in a narrow band (approximately 8μm wide). It is possible that the total amount of medullary bone mineralisation in the mid-lay group was similar to that in the [1egg] group, but that it occurred at a greater number of sites and in a more concentrated fashion due to the vastly increased volume of medullary bone present.

Ultrastructural changes observed were generally consistent with those seen at light microscope level. They were also similar to descriptions of bone-lining cell and osteoclast activation after oestrogen administration (Bowman & Miller, 1986; Miller, 1988, 1990).

Young osteocytes which have become newly embedded in their own matrix resemble osteoblasts; their nucleus has prominent nucleoli, they have extensive rough endoplasmic reticulum and golgi apparatus and mitochondria are present throughout the cytoplasm. As the

cell becomes more deeply embedded in the bone the cell becomes smaller, at the expense of the cytoplasm (which retains all the cell organelles), the nucleus becomes the prominent feature, and the lacuna in which the osteocyte is situated becomes smaller. These changes can be regarded as formative stages in the osteocyte life cycle, and osteocytes of this appearance are characteristic of new bone (Tonna, 1973; Holtrop, 1990). In bone tissue where osteocytes have a longer life span, for example in the osteons of compact bone of adult mammals, further changes in osteocyte morphology are recognised. The osteocyte progresses to a resorptive phase, in which little rough endoplasmic reticulum remains but the golgi apparatus remains present, though in diminished quantities. The frequency of lysosomes is also increased. The final stage is a resorptive stage which utimately results in cell death. These morphological changes are characteristic of ageing tissue and are found deep within the bone. They have also been associated with the controversial process of osteocytic osteolysis, which is a resorptive function of osteocytes reported to occur under certain metabolic conditions in man (Belanger, 1969; Jande et al, 1973). This process has also been observed during antler formation in reindeer and in laying hens (Belanger et al, 1967; Taylor & Belanger, 1969). The opposing view, however, is that such morphological changes occurring in deeply embedded osteocytes are simply fixation artifact (Holtrop, 1990)

In the present experiment differences in morphology between the osteocytes of medullary and cancellous bone were noted. Degenerative changes were observed in deeply embedded cancellous bone osteocytes but were never seen in medullary bone osteocytes, which consistently had the appearance of cells in the formative stage. These differences may simply be a reflection of the accelerated rate of turnover in medullary bone compared with that in cancellous bone. It is possible that the osteocytes of medullary bone do not exist long enough to undergo degenerative changes.

The whole activation, resorption and formation sequence of bone remodelling in mammals occurs in small areas (<20% of the available bone surface), with a cyclical timing and slow apposition rate (0.3- 1.0μm/day). In contrast bone modelling occurs without coupling of formation to resorption, since there tends to be more formation than resorption. Also, it

occurs over >90% of the available bone surface and at a fast apposition rate of 2-10μm/day (Jee, 1988). The activation, resorption and formation which occurs in the bones of laying hens during reproductive development and the subsequent repeated, cyclical resorption and mineralisation that occurs during the egg-formation cycle do not fit neatly into either category. However, it was considered appropriate to use the term modelling to describe the process of medullary bone formation that occurs during ovarian follicular development because it occurs over most of the bone surfaces, at a fast rate and results in the resorption of cancellous bone and its replacement with woven bone. The resorption and formation of medullary bone once egg-laying has commenced occurs cyclically and at discrete sites and is most appropriately described as remodelling.

In the present study it was found that cancellous bone undergoes a significant reduction in volume during medullary bone modelling. This coincides with the observations made on histological sections; firstly, at the onset of follicular development there was a simultaneous increase in osteoclast number and plumping of the bone lining cells. At the next stage of follicular development there was a measured decrease in cancellous bone volume, which continued through to the point of lay. This period represents the process of medullary bone modelling, during which medullary bone is formed but not subjected to the daily periods of intense osteoclastic resorption induced by egg-shell formation.

In the birds used in this experiment, medullary bone volume was relatively low at the start of lay (2.67%), and consisted of small spicules confined to cancellous and endocortical surfaces.

The volume of medullary bone in the humerus was higher at the onset of lay than in the tarsometatarsus, but insignificantly increased in volume during remodelling. This may have been because of the variability in medullary bone volume in the humerus, which in turn is largely due to the degree of pneumatisation in each individual. In birds whose air sac does not extend into the extremities of the humerus, medullary bone volume is higher than in those birds with a fully extended air sac. It is also possible that the presence of the air sac (or the absence of bone marrow) in some way limits the development of medullary bone. The air sac

epithelium may act as a physical barrier to the development of medullary bone and the absence of bone marrow may affect recruitment of osteogenic cells.

Cortical bone thickness of birds in the present study was unaffected by the process of medullary bone modelling. Cancellous bone has a higher surface to volume ratio than cortical bone, and alterations in remodelling dynamics usually exert a greater influence on the former (Marcus, 1987). Cancellous bone is therefore a more sensitive indicator of change in the balance of formation and resorption, and may therefore explain the differences between the effects of medullary bone modelling on cancellous and cortical bone. In the present experiment, cancellous bone volume in the tarsometatarsus diminished further during medullary bone remodelling, while medullary bone volume increased dramatically. In the Bell & Siller study (1962) the compacta of pre-lay pullets was described as thick, but that in heavily laying normal pullets they were thinned and had extensive resorption cavities and there was extensive diminution of medullary bone trabeculae. In birds with cage layer fatigue this was exaggerated, but no attempt was made to quantify the thinning. In hens fed low calcium diets, medullary bone is maintained at the expense of cortical bone, which undergoes increased resorption on endocortical surfaces and in severe deficiency, increased intracortical resorption (Urist, 1959; Simkiss, 1967). The cortices of hens in this experiment were observed to have undergone both intracortical remodelling and cancellisation during medullary bone remodelling. There was no evidence of nutritional deficiency, since the medullary bone trabeculae were well mineralised. The loss of both cortical and cancellous bone during the reproductive period may be regarded as normal in the modern laying hen. Peak bone mass therefore occurs before follicular development.

Within the [NFD] group, the older birds appeared to have higher cancellous bone volumes than the younger birds, but the small group size renders this observation unreliable as an indicator of the effects of age on this parameter. It is possible that a longer period of consolidation may result in an increased peak bone mass, and this provides an interesting area for further experiments. In man, this period of consolidation continues into the third decade, obviously beyond puberty. The sequence of events occurring in the bones of pullets during reproductive development suggest that consolidation of structural bone in the laying hen is

interrupted by the process of medullary bone formation.

It is also interesting to note that the sum of cancellous and medullary bone mid-lay is greater than the volume of cancellous bone before the onset of follicular development; the laying hen has therefore accumulated bone in the marrow cavity during this period. However, the replacement of lamellar bone with woven bone (even in larger quantities), is unlikely to enhance the mechanical competence of the skeleton.

The mechanical competence of the hen skeleton may be further compromised by the absence of the type of lamellar remodelling which occurs in male birds and in mammals. Remodelling in mammals comprises discrete osteoclastic resorption followed by osteoblastic replacement of bone at the same site. In the laying hen, by mid-lay, bone resorption and formation are restricted to medullary bone, the cancellous bone trabeculae being covered by deep seams of medullary bone. Remodelling of lamellar bone is necessary to replace osteocytes which have undergone degenerative changes and death as the ultimate end of their life cycle (Tonna, 1973). The effects of an increasing population of unremodelled degenerating cancellous bone osteocytes on the hen skeleton's mechanical competence are unknown, but unlikely to be positive.

The changes in bone mass which occur in the laying hen can be summarised as follows:

1. Medullary bone formation is preceded by an activation of bone lining cells and osteoclasts, which coincides with an initial rise in plasma oestradiol.

2. Cancellous bone loss occurs throughout medullary bone modelling, commencing in the initial stages of ovarian follicle development.

3. Medullary bone trabeculae are modelled during the final growth phase of the developing ovarian follicles, when oestrogen secretion peaks.

4. Medullary bone remodelling during the egg-laying cycle results in its volume increasing markedly.

5. Cancellous bone loss continues during medullary bone remodelling.

6. Cortical bone loss occurs primarily during medullary bone remodelling.

This pattern of structural bone loss is clearly very different to that which occurs in mammals. While the mammalian skeleton loses bone and increases its rate of remodelling in response to oestrogen loss (Aitken et al, 1973; Richelson et al, 1984), the skeleton of the modern laying hen (and possibly all avians) responds to the presence of oestrogen by increasing the rate of remodelling and decreasing the volume of structural bone.

The skeletal effects of oestrogen

Oestrogen has a profound effect on bone, promoting major skeletal effects such as inhibition of linear growth, acceleration of skeletal maturation through epiphyseal closure, and increased density of bone (Turner & Schraer, 1977). Further evidence of the importance of oestrogen in bone metabolism is indicated by the loss of bone mass in women following the menopause, and oestrogen is used extensively in the prevention and treatment of post-menopausal osteoporosis (Linsay & Cosman, 1992). The inhibitory effect of oestrogen on bone resorption has been repeatedly demonstrated *in vivo* but not *in vitro* at physiological levels (Caputo et al, 1976). These results were thought to indicate that the effects of oestrogen on bone were indirect (Raisz, 1992), but recent evidence indicates that oestrogen directly inhibits resorption and stimulates formation (Takano-Yamamoto & Rodan, 1990).

Classic oestrogen target organs such as uterus, oviduct and liver contain specific receptors that form high affinity complexes with the hormone (Toft & Gorski, 1966). Early attempts to identify oestrogen receptors in rat bone were unsuccessful (Nutik & Cruess, 1974; Chen & Feldman, 1978), but more recent studies have demonstrated estrogen receptor-like binding in cultured human bone cells (Eriksen et al, 1988). The primary target cells for oestrogen in mammalian bone are not known.

The natural response of the avian skeleton to oestrogen is the formation of medullary bone, and the previous chapter described the bone changes which occur during the course of ovarian follicular development and the subsequent egg laying cycle in hens. Clearly, the presence of oestrogen is associated with the development of osteoporosis in the modern egg laying fowl, and this is in complete contrast with the development of osteoporosis in mammals, which is commonly attributed to the absence of oestrogen.

Although medullary bone is naturally confined to reproductive female birds, it can be readily induced in male birds by the administration of oestrogen (Pfeiffer & Gardner, 1938; Bloom et al, 1942; Landauer & Zondek, 1954; Simmons & Pankovich, 1964). Oestrogen-treated male birds have been used extensively to study osteogenesis in the adult skeleton because they present an orderly and predictable sequence of osteogenic events (Bowman & Miller, 1986).

In a study of oestrogen-induced sequential changes in quail bone metabolism (Turner & Schraer, 1977), it was found that the organic weight of the femur increased after 36 hours, and ash weight after 96 hours. Collagen formation began to increase 36 hours after oestrogen administration, and reached a peak of 3.5 times the rate in control animals after 60 hours. Matrix mineralisation began approximately 24 hours after the onset of collagen synthesis. Medullary bone trabeculae greater than 1000μm in thickness were observed after 72 hours.

Histological studies have described the sequence of cellular changes in bone which follow oestrogen administration in male quail (Millar & Bowman, 1981; Bowman & Millar, 1986). In untreated controls, 77% of endosteal surfaces of the femoral diaphysis were covered by low-density populations (21 cells/mm) of flattened bone lining cells. 24 hours after oestrogen administration, these surfaces were covered, at a density of 38 cells/mm, by plump cells with abundant polyribosomes and large oval or round nuclei. By 36 hours these cells had extensive rough endoplasmic reticulum and Golgi complexes, and extracellular medullary bone matrix was visible. Medullary bone trabeculae were evident 48 hours after oestrogen administration as was some mineralisation. The developing bone grew rapidly into the marrow cavity between 72 and 120 hours post-injection, and medullary bone modelling was accompanied by dramatic increases in total plasma calcium levels. This sequence of cellular changes in response to oestrogen has become known as the "endosteal reaction".

Kusuhara & Schraer (1982), measured ^{3}[H]thymidine labelling of endosteal cells during the "endosteal reaction", and found it to be maximal 27 hours after oestrogen administration. Preosteoblasts and osteoblasts were labelled 7 and 10 hours later, respectively. The authors

concluded that under the influence of oestrogen, endosteal cells maximally synthesize DNA after 27 hours and modulate into preosteoblasts in approximately six hours then divide to become osteoblasts within a further 3 hours.

Immunohistochemical detection of oestrogen receptors in bone lining cells, preosteoblasts, and osteoblasts has been reported during oestrogen-induced medullary bone modelling in male quail (Oshashi et al, 1991), suggesting oestrogen acts directly on medullary bone osteogenesis. A similar study by Turner et al (1993) indicated that bone contains less than 8% of the oestrogen binding sites found in oviduct.

Only one of the studies involving oestrogen-induced medullary bone modelling has examined the subsequent effects on structural bone. Turner et al (1993) demonstrated that in oestrogen-treated male Japanese quail, medullary bone formation and hypercalcaemia were accompanied by a reduction in cortical bone area. Cancellous bone volume was not measured, however. The results from the first experiment in the present study indicated that cortical and cancellous bone loss occurred during natural medullary bone modelling.

The sketetal effects of anti-oestrogens

Tamoxifen is one of the anti-oestrogenic compounds developed initially for use as contraceptives then later used for infertility treatment (Anon., 1983). Anti-oestrogens compete with oestrogens by binding to cytoplasmic receptor proteins, and tamoxifen is now used extensively for the treatment for breast cancer (Tagnon,1977; Nayfield et al, 1991). Its effects on the skeleton were considered worthy of study both because of the duration of treatment and the fact that a high proportion of sufferers are post-menopausal (Furr et al, 1978; Davidson, 1992).

Tamoxifen can behave as an oestrogen agonist, a partial agonist, or as an antagonist, depending on the species studied, the target organ assessed, and the dose in relation to the

oestrogen concentration (Legha, 1988). Thus in premenopausal women treated for breast cancer, tamoxifen is thought to act as an oestrogen antagonist in bone because it reduces bone mineral content of the forearm (Gotfredsen et al, 1984). In post-menopausal women, however, tamoxifen treatment results in increased bone mineral density (Turken et al,1989; Love et al, 1992) or increased bone mineral content (Ryan et al, 1991), and therefore appears to act as an oestrogen agonist under these circumstances. Results from rat studies support this; tamoxifen has a protective effect on bone in ovariectomised animals (Turner et al, 1987; Wakeley et al, 1987; Turner et al, 1988) but adverse skeletal effects in sexually mature females (Feldman et al, 1989).

Turner et al (1987) reported that the protective effect of tamoxifen on the skeleton of ovariectomised rats was partially mediated by inhibition of osteoclast number and activity. The mechanisms of this action are not known, but high concentrations of tamoxifen inhibit PTH-stimulated bone resorption in vitro, and are cytotoxic to osteoclasts (Stewart & Stern, 1986; Arnett et al, 1986).

The effects of tamoxifen on the avian reproductive system and on medullary bone formation have also been investigated. Jaccoby et al (1992), administered various doses of tamoxifen to 2 week-old White Leghorn hens on alternate days for a period of 21 weeks. Tamoxifen caused a precocious increase in plasma oestrogen and androgen and decreased adiposity from 6 weeks of age. At 23 weeks of age the gonads of controls were fully mature and active but at doses between 1 and 10mg/kg body weight tamoxifen depressed plasma calcium, weights of the liver, oviduct, and ovary, and maintained increased plasma oestrogen concentrations. However, at doses of 1mg/kg or less, tamoxifen advanced egg laying. Tamoxifen, therefore, appears to have both oestrogenic and antioestrogenic gonadal effects, which are dose dependant (Rozenboim et al, 1989).

Oshashi et al (1987, 1988) demonstrated the typical "endosteal reaction" in oestrogen-treated male quail, resulting in the deposition of medullary bone at the endosteal bone surface after 36 hours. However, after oestrogen and tamoxifen treatment the numbers of preosteoblasts

and osteoblasts on the endosteal surface did not increase, but slowly decreased in number while osteoclast numbers increased. Alkaline phosphatase activity was demonstrated on the cell membranes of endosteal cells, preosteoblasts and osteoblasts of oestrogen-treated birds but was absent in those cells of oestrogen and tamoxifen-treated birds. There was also no medullary bone formation.

Williams et al, (1991) carried out a study of the effects of tamoxifen on oestrogen-treated quail medullary bone in order to determine whether it was selectively agonistic or antagonistic. The administration of tamoxifen alone to male quail did not stimulate medullary bone formation or affect serum calcium and phosphorus levels or alkaline phosphatase activity. Oestrogen treatment resulted in medullary bone formation, reduced aggression and testicular atrophy, and increased serum calcium, phosphorus and alkaline phosphatase. Oestrogen and tamoxifen treatment inhibited medullary bone formation and the corresponding increases in serum calcium, phosphorus and alkaline phosphatase activity. The authors concluded that tamoxifen acted as a pure oestrogen antagonist with respect to medullary bone formation in Japanese quail.

Aims of the experiment

Although oestrogen-induced medullary bone formation in male birds has been widely investigated, its subsequent effects on structural bone loss are unknown. The anti-oestrogenic effects of tamoxifen on oestrogen-induced medullary bone formation in male quail have been demonstrated, as have its mixed effects on gonadal development in female fowl. The effects of tamoxifen on medullary bone modelling and subsequent structural bone loss are not known in the laying hen. Tamoxifen will be administered to females during the normal period of ovarian follicular development, and oestrogen will be administered to male fowl of the same age. Histomorphometric analysis of bone samples collected from these birds will be carried out in order to determine the role of oestrogen in structural bone loss in laying strain fowl.

Animals

50 female and 50 male day-old Hisex chicks (Ross Poultry) were housed separately in brooders until 4 weeks of age then transferred to individual cages. They were fed standard rations ad libitum. At fourteen weeks of age, they were divided into four groups as follows: control females (n=24); tamoxifen-treated females (n=26); control males (n=25); oestrogen-treated males (n=25). Tamoxifen-treated females received on, alternate days, 10mg/kg tamoxifen (Sigma) intra-muscularly for a four week period. Control females were similarly treated, but with vehicle alone. Oestrogen-treated males received 20mg/kg β–oestradiol 17-valerate (Sigma) in corn oil intra-muscularly at weekly intervals for two weeks from 16 weeks of age. Control males were similarly treated with corn oil alone. Intravenous fluorochrome labels were administered to all birds before sacrifice at 18 weeks of age as follows; 25mg/kg oxytetracycline (Engemycin, Mycofarm UK Ltd), 72 hours and 20mg/kg fluorescein complexone (BDH Laboratory Supplies) 96 hours. Blood samples were collected immediately before sacrifice. Carcasses were weighed prior to dissection.

Blood Chemistry

Plasma calcium and oestradiol were measured as described in Experiment 2.

Bone samples

The right proximal tarsometatarsus and tarsometatarsal diaphysis were removed from each bird, trimmed and processed for decalcified sections. The corresponding samples from the left appendicular skeleton were processed for undecalcified sections. $1mm^3$ pieces from the marrow cavity of the proximal femur were processed for electron microscopy. Processing and staining were carried out according to the schedules described in Experiment 1.

Histomorphometry

Histomorphometric measurements were performed as described in Experiment 2.

Statistical Analysis

This was also carried out as described in Experiment 2.

General Appearance and Body Weight

All of the birds used were observed daily during the course of the experiment, and the control and experimental female birds and the control male birds were considered healthy. The oestrogen-treated male birds, however, began to appear subdued after the first week of treatment, and at the time of sacrifice had lost their comb colour (Figure 25).

Body weight of all birds are shown in Table 7, and mean values for each group in Table 12. There was no significant difference in mean body weight between control and experimental females, or control and experimental males.

Examination of reproductive tract of female birds indicated that normal follicular development was inhibited in tamoxifen-treated birds; there was no follicular development in three of the birds while in the remainder there was some follicular development, the largest follicle recorded being 12mm in diameter. In contrast, the control group consisted of birds with a range of follicular development, from one bird with no follicular development through to eleven in which egg laying had commenced.

Blood Chemistry

Total calcium values of all birds are shown in Table 8, and mean values for each group in Table 12. Total plasma calcium was significantly lower ($p<0.001$) in tamoxifen-treated females than in control females, and was significantly higher ($p<0.001$) in oestrogen-treated males than control males.

Plasma oestradiol values of all birds are shown in Table 9, and mean values for each group in Table 12. Oestradiol values were significantly higher ($p<0.001$) in tamoxifen-treated females than control females, and in oestrogen-treated males compared with control males.

Figure 25. Control (left) and oestrogen-treated (right) cockerels.

Histology

Control females

Decalcified sections of proximal tarsometatarsus reflected each animal's state of follicular development. Those in the early stages of follicular development (<9mm) had no medullary bone and cancellous bone with the characteristic beading of activated bone lining cells and numerous osteoclasts. Most of the birds were in lay and therefore had extensive medullary bone development along cancellous and endocortical bone surfaces. These medullary bone spicules were associated with groups of osteoblasts, and numerous osteoclasts were observed adjacent to both cancellous and medullary bone.

Undecalcified stained sections indicated normally mineralised bone; narrow seams of cancellous bone osteoid were present in birds with no or very early stages of follicular development, while cancellous bone trabeculae of birds in lay were fully mineralised and medullary bone spicules were fringed by very fine osteoid seams.

The cortices were of normal thickness and general appearance; they were similar to the birds in comparable reproductive states described in Experiment 2.

Fluorochrome labelling also reflected follicular development; both labels were present as extensive narrow bands in cancellous bone in birds with no or very early stages of follicular development. Those birds in lay had both cancellous and medullary bone labelling.

Tamoxifen-treated females

Decalcified proximal tarsometatarsal sections differed from most of the control group in that there was a complete absence of medullary bone. The cancellous bone trabeculae were thick and well connected, extending throughout the marrow cavity. The bone-lining cells which covered their surfaces were typical of the early stage of follicular development; they were slightly more rounded in appearance than in birds with no follicular development. Although osteoclasts were observed, none were located in resorption cavities or in close adherence to the bone surfaces.

The cortices appeared normal, and differed from the control birds only in the absence of medulary bone on the endocortical surfaces.

Cancellous bone osteoid seams occurred occasionally in undecalcified stained sections of proximal tarsometatarsus, and were similar to those osteoid seams seen in control females with no follicular development.

Fluorochrome labelling was less extensive than in control females with no follicular development, both labels occurring as very close mostly parallel bands in the cancellous bone trabeculae.

Control males

The male birds in this group all had thick tarsometatarsal cortices and an extensive network of thick cancellous bone trabeculae in the proximal tarsometatarsus. The cancellous bone surfaces were mostly covered with bone lining cells but regular groups of active osteoblasts were observed. Several osteoclasts were observed within deep resorption lacunae.

Undecalcified stained sections indicated normally mineralised cancellous bone trabeculae, with regular seams of unmineralised matrix. Examination of unstained 12μm sections under transmitted ultra violet light revealed the extensive presence of both the fluorochrome labels, which both occurred as narrow bands, parallel to, and beneath the cancellous bone surface.

Oestrogen-treated males

Decalcified sections indicated that oestrogen treatment had been successful in inducing medullary bone modelling in all of the treated male birds. The cancellous bone trabeculae appeared fragmented and thin, compared with that of control males and their surfaces in areas without medullary bone modelling were covered mainly by a layer of quiescent bone lining cells. Areas of medullary bone modelling were associated with intensive osteoclastic and osteoblastic activity. The medullary bone osteocytes appeared larger than their cancellous bone counterparts. In ten of the birds in this group, medullary bone was present in tiny spicules only in the diaphysis, and therefore resulted in zero medullary bone counts of the proximal tarsometatarsus. In those birds with more widespread medullary bone development, there were two forms present. Medullary bone in the form of spicules modelled onto the cancellous bone surface was present in some birds, while in others it took the form of long narrow bands along the cancellous bone surface. The latter type was difficult to detect in

decalcified sections stained with haematoxylin and eosin, but was more obvious in toluidine blue stained undecalcified sections. The medullary bone in these sections examined under polarised light failed to exhibit the birefringency characteristic of lamellar bone, indicating its woven nature.

Fluorochrome labelling occurred either as two diffuse medullary bone labels or the first label as a narrow band in cancellous bone and the second as a diffuse medullary bone label. This appearance was similar to control, in-lay females.

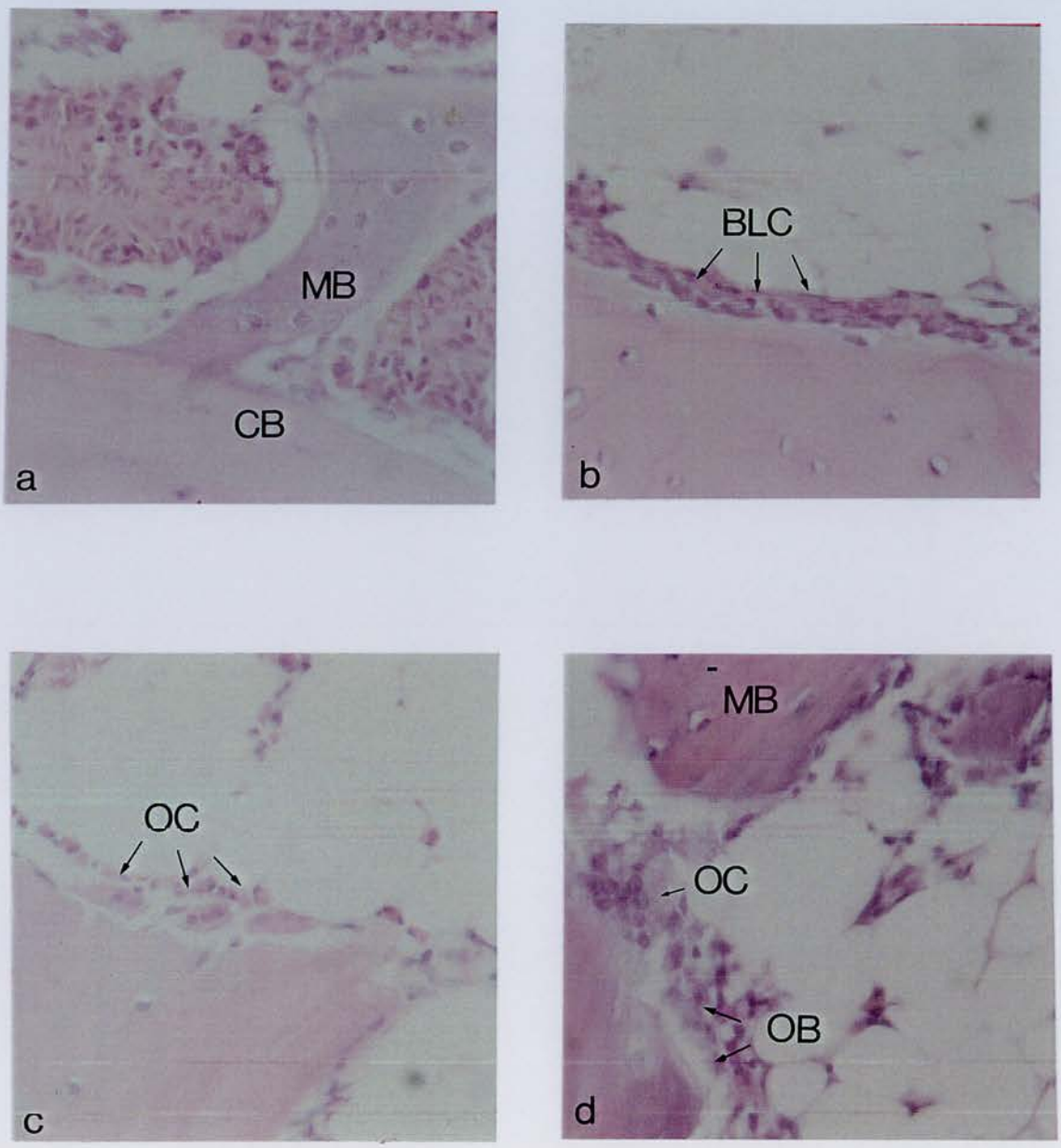


Figure 26. Decalcified sections of proximal tarsometatarsus stained H&E. a) control female (x 470) b) tamoxifen-treated female (x 470) c) control male (x 470) and d) oestrogen-treated male (x 470). CB-cancellous bone MB-medullary bone BLC-bone lining cell OB- osteoblast OC-osteoclast

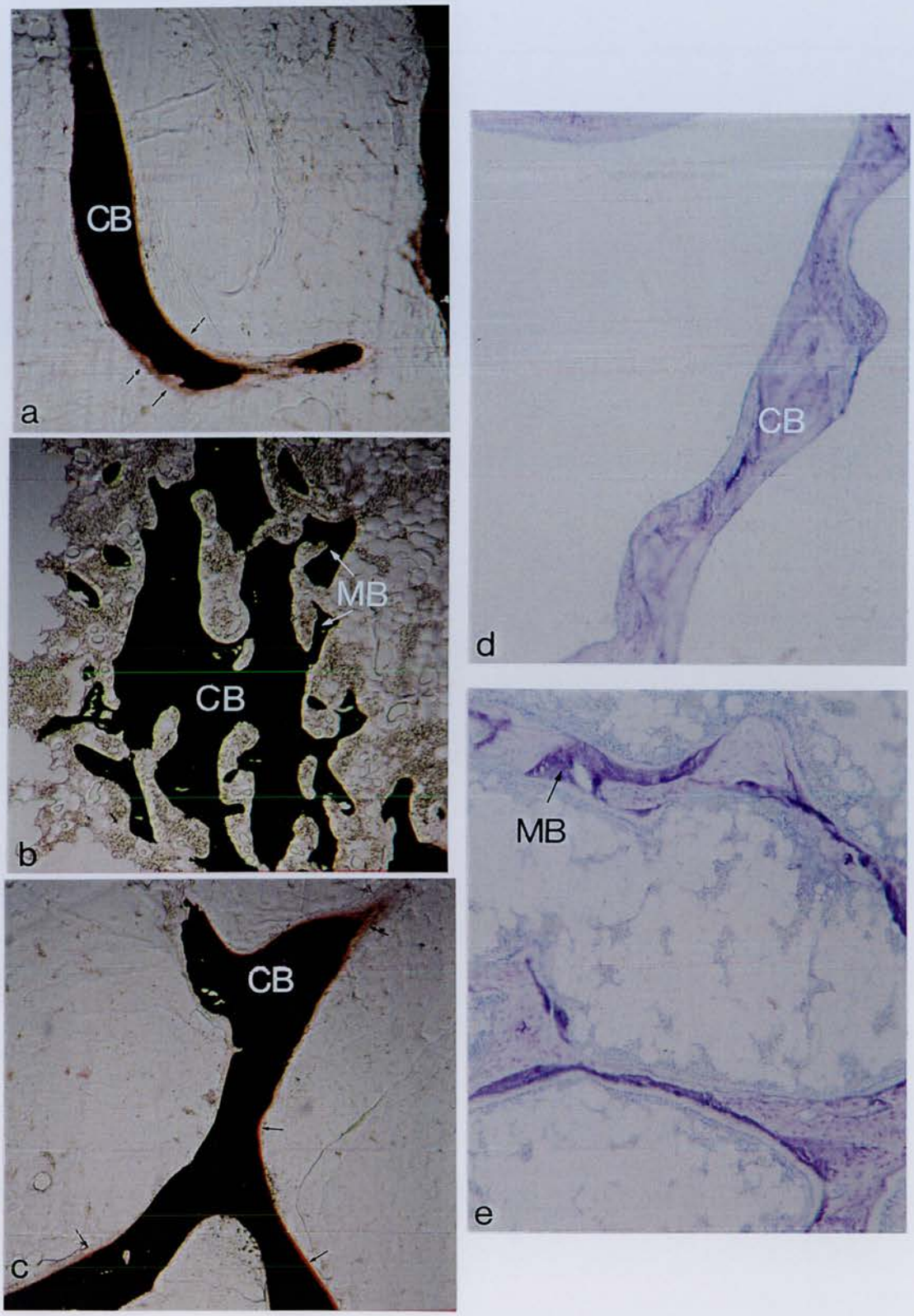


Figure 27. Undecalcified sections of proximal tarsometatarsus stained Von Kossa (a-c) or tol. blue (d, e). a)tamoxifen-treated female b)oestrogen-treated male c) control male d) control male and e) oestrogen-treated male. MB-medullary bone; CB-cancellous bone; arrows-unmineralised matrix (x240)

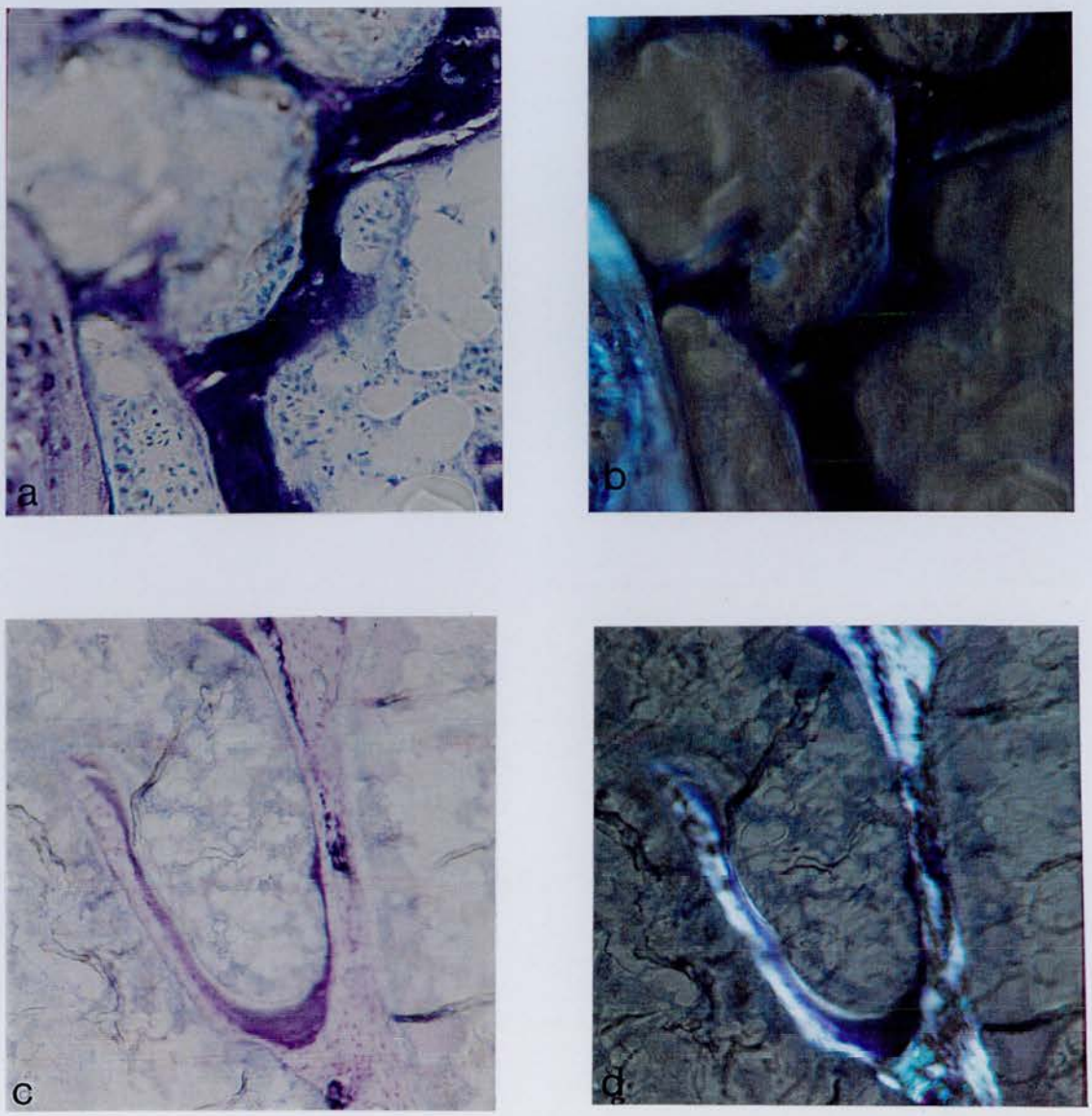


Figure 28. Undecalcified tol. blue stained sections under normal and polarised light. a)control female (x470) b)oestrogen-treated male (x240). CB-cancellous bone MB-medullary bone

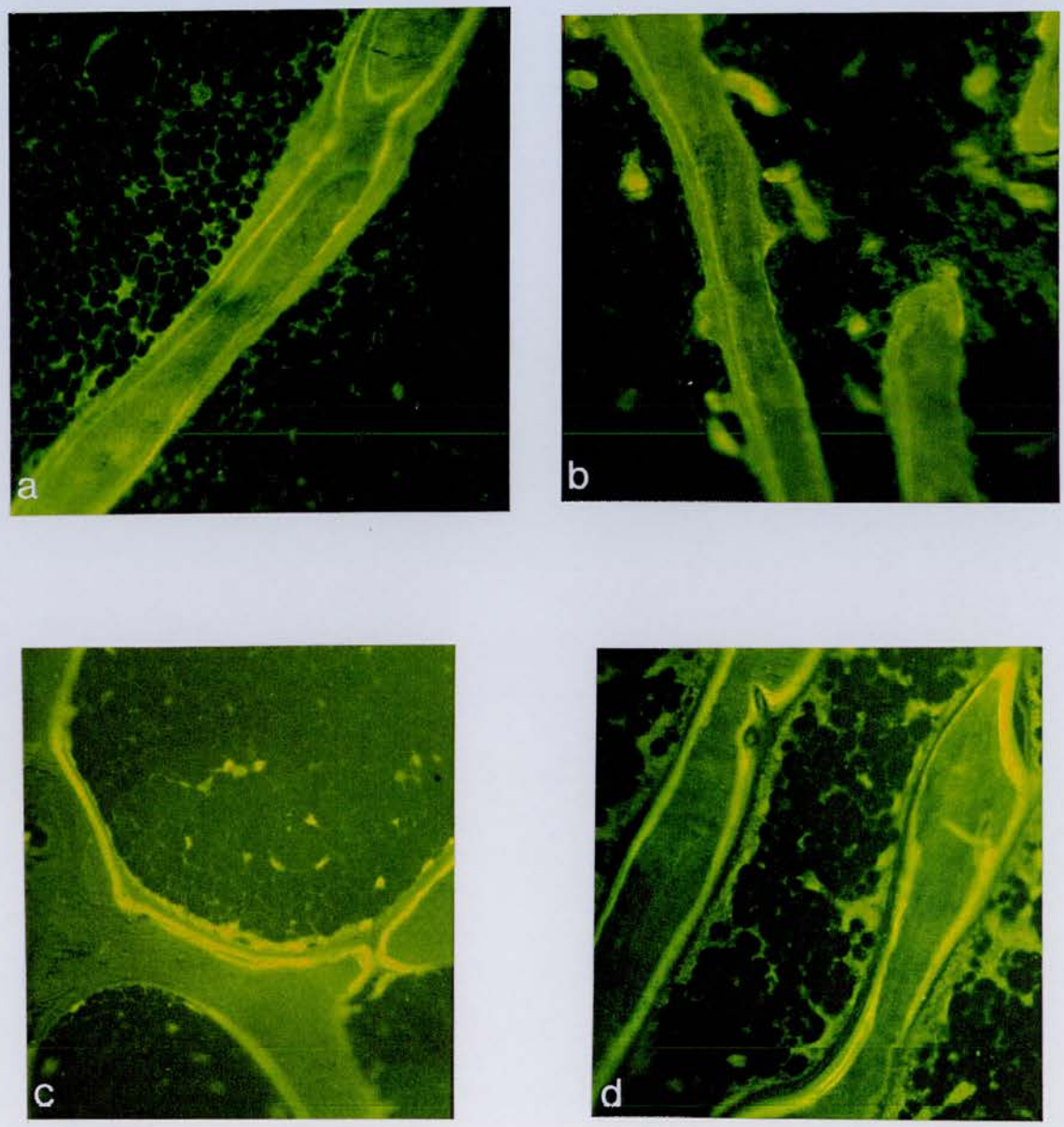


Figure 29. Unstained undecalcified sections of proximal tarsometatarsus labelled with oxytetracycline (OTC) and fluorescein complexone (FC). a)control female b) control female c) tamoxifen-treated female d)control male (x6.3)

Ultrastructure

Control females

The ultrastructural appearance of the control females was similar to that described for the birds in Chapter 2, and was related to the individual's state of follicular development. The differences between cancellous (Figure 30), and medullary bone osteocytes (Figure 31) were again very obvious.

Tamoxifen-treated females

The cancellous bone surfaces were covered by bone lining cells which appeared to be partially activated; they were not cuboidal, like active osteoblasts, but were plumper than bone lining cells.The nucleus was no longer stretched along the bone surface, but appeared irregular and slightly compressed in shape, while the rough endoplasmic reticulum was excessively dilated (Figure 32). No osteoclasts were observed in contact with the bone surface. There was no medullary bone, and the cancellous bone osteocytes were similar in appearance to those in the control female group.

Control males

The cancellous bone surfaces of control males were covered by bone lining cells and occasional groups of active osteoblasts. In one area, these were observed to be in the process of embedding themselves in new bone matrix (Figure 33). No osteoclasts were observed in contact with the bone surface. Osteocytes were typical for cancellous bone.

Oestrogen treated males

Many active osteoblasts (Figure 34) were observed on the cancellous bone surface. These were rounded in appearance, with dilated rough endoplasmic reticulum. In all other respects the bone was also similar to the control females in advanced stages of follicular development.

Figure 30. Cancellous bone osteocyte from control female (x 16200)

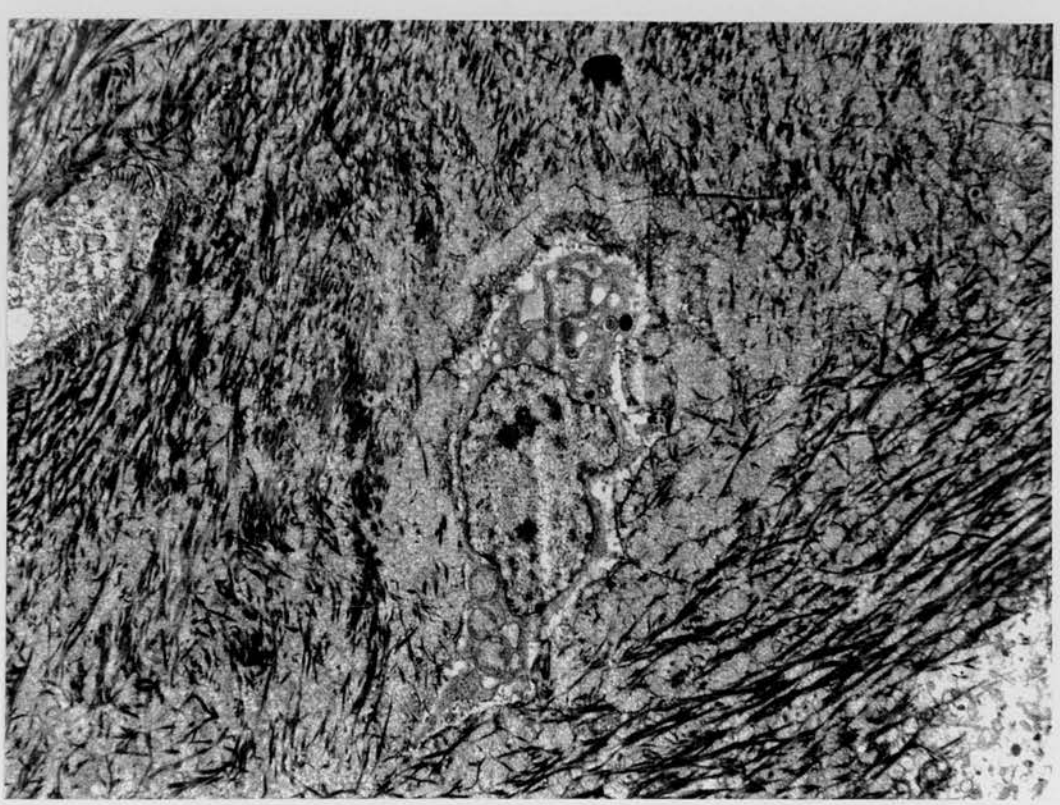


Figure 31. Medullary bone osteocyte from control female (x 4374)

Figure 32. Partially activated bone lining cell from tamoxifen-treated female (x 6156) RER- rough endoplasmic reticulum

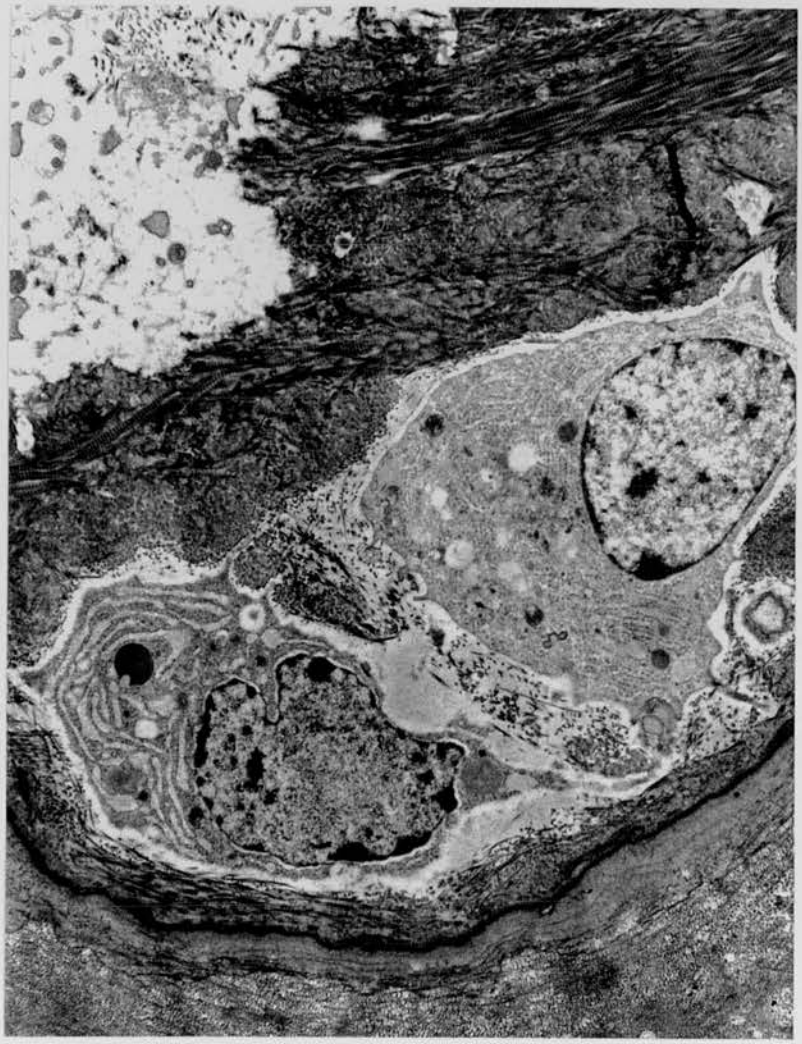


Figure 33. Newly formed osteocytes from the femur of a control male bird (x 6156)

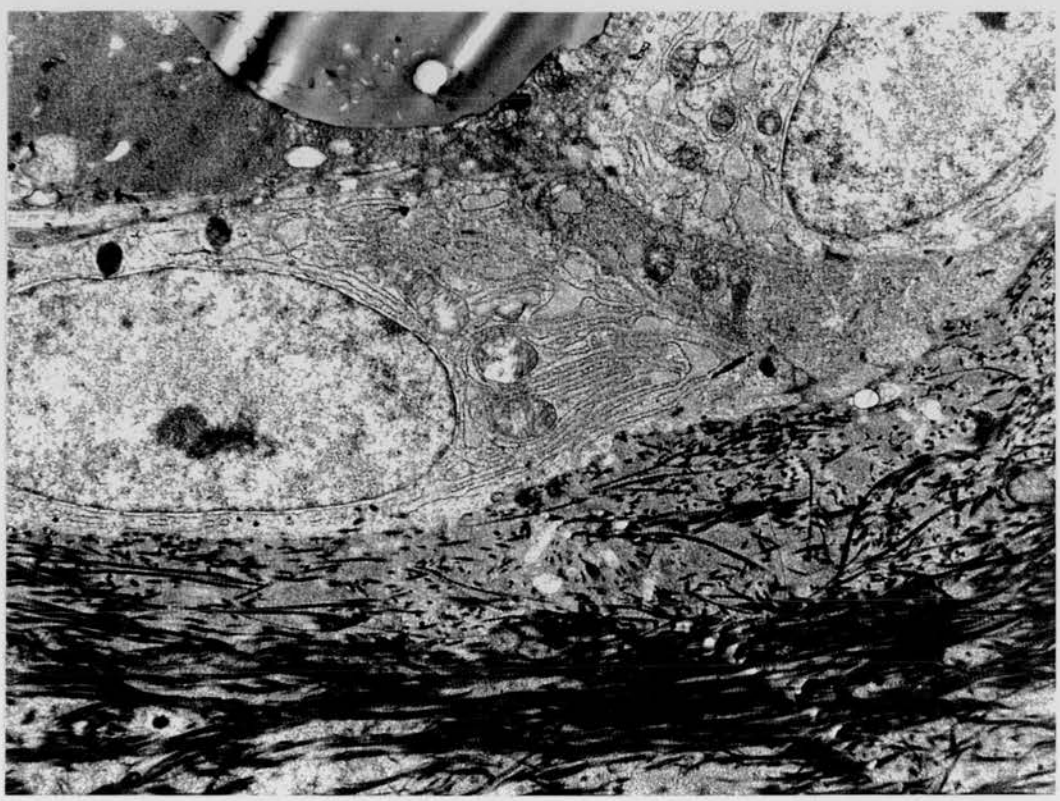


Figure 34. Activated bone lining cells on the cancellous bone surface, from the femur of an oestrogen-treated male bird (x 5022)

Histomorphometry

Proximal tarsometatarsal cancellous bone volumes and medullary bone volumes of all birds are shown in Table 10, tarsometatarsal diaphysis cortical thickness measurements in Table 11, and mean values for each group in Table 12.

Cancellous bone volume was significantly greater ($p<0.001$) in tamoxifen-treated females than in control females. Medullary bone modelling was completely prevented in tamoxifen-treated birds, resulting in a highly significant difference in medullary bone volume between them and controls. In contrast, oestrogen-treated male birds had significantly lower ($p<0.001$) cancellous bone volumes than control males. Medullary bone modelling was successfully induced by the oestrogen treatment, resulting in a significant difference in medullary bone volume between these and the control males. There was no significant difference in cortical thickness between either the tamoxifen-treated and control females, or between the oestrogen-treated and control males.

Table 7. Body Weights (kg)

control ♀'s		exp ♀'s		control ♂'s		exp ♂'s	
bird no.	body weight	bird no.	body weight	bird no.	body weight	bird no.	body weight
1623	1.60	1648	1.72	1673	2.70	1698	2.80
1624	1.70	1649	1.68	1674	2.46	1699	2.70
1625	1.70	1650	1.68	1675	2.40	1700	2.80
1626	1.78	1651	1.72	1676	2.66	1701	2.38
1627	1.46	1652	1.76	1677	2.34	1702	2.68
1628	1.96	1653	1.78	1678	2.90	1703	2.88
1629	1.64	1654	1.74	1679	2.58	1704	3.14
1630	1.78	1655	1.74	1680	2.70	1705	2.80
1631	2.00	1656	1.86	1681	2.88	1706	2.64
1632	1.92	1657	1.96	1682	2.92	1707	2.64
1633	1.80	1658	1.70	1683	2.50	1708	2.76
1634	2.44	1659	1.80	1684	3.16	1709	2.68
1635	2.10	1660	1.90	1685	2.62	1710	2.88
1636	1.78	1661	1.94	1686	2.62	1711	2.26
1637	1.90	1662	1.82	1687	2.70	1712	3.60
1638	1.80	1663	1.98	1688	2.70	1713	3.02
1639	1.64	1664	2.08	1689	2.60	1714	3.26
1640	1.90	1665	1.98	1690	2.42	1715	2.86
1641	1.96	1666	1.78	1691	2.86	1716	2.40
1642	1.74	1667	1.74	1692	2.78	1717	1.50
1643	2.00	1668	1.84	1693	2.46	1718	2.86
1644	1.76	1669	1.90	1694	2.68	1719	2.68
1645	1.74	1670	1.94	1695	2.52	1720	2.56
1646	1.84	1671	1.78	1696	2.92	1721	2.70
		1672	1.85	1697	2.88	1722	2.58
		1647	1.78				

Table 8: plasma calcium (mmol/l)

control ♀'s		exp ♀'s		control ♂'s		exp ♂'s	
bird no.	plasma Ca	bird no.	plasma Ca	bird no.	plasma Ca	bird no.	plasma Ca
1623	3.74	1648	0.71	1673	0.78	1698	8.11
1624	0.98	1649	0.81	1674	0.65	1699	1.24
1625	0.53	1650	0.89	1675	1.43	1700	1.94
1626	1.22	1651	1.64	1676	0.72	1701	1.17
1627	3.53	1652	1.71	1677	0.64	1702	8.22
1628	*	1653	*	1678	1.02	1703	10.32
1629	3.93	1654	0.97	1679	0.72	1704	0.89
1630	4.77	1655	0.84	1680	0.93	1705	0.57
1631	6.95	1656	1.13	1681	1.09	1706	0.94
1632	7.87	1657	1.27	1682	0.82	1707	*
1633	4.51	1658	2.54	1683	*	1708	0.94
1634	4.97	1659	3.93	1684	0.80	1709	1.12
1635	*	1660	2.13	1685	*	1710	8.72
1636	*	1661	1.41	1686	0.84	1711	9.38
1637	4.20	1662	1.57	1687	0.73	1712	7.99
1638	1.21	1663	0.86	1688	0.99	1713	8.65
1639	1.19	1664	0.99	1689	0.94	1714	8.13
1640	4.26	1665	*	1690	1.14	1715	8.42
1641	5.76	1666	2.12	1691	1.16	1716	1.38
1642	3.37	1667	1.18	1692	1.10	1717	7.70
1643	4.14	1668	0.98	1693	1.12	1718	8.78
1644	*	1669	0.90	1694	0.83	1719	8.97
1645	*	1670	1.12	1695	1.23	1720	8.08
1646	*	1671	1.22	1696	0.77	1721	8.94
		1672	1.26	1697	*	1722	0.64
		1647	0.71				

* missing value

Table 9. Plasma oestradiol (pg/ml)

control ♀'s		exp ♀'s		control ♂'s		exp ♂'s	
bird no.	plasma oest.	bird no.	plasma oest.	bird no.	plasma oest.	bird no.	plasma oest.
1623	383	1648	1692	1673	23	1698	128
1624	103	1649	625	1674	26	1699	*
1625	195	1650	1544	1675	30	1700	138
1626	217	1651	704	1676	22	1701	289
1627	119	1652	952	1677	23	1702	176
1628	*	1653	994	1678	9	1703	889
1629	171	1654	*	1679	22	1704	2440
1630	93	1655	470	1680	51	1705	69
1631	185	1656	*	1681	25	1706	74
1632	211	1657	1115	1682	24	1707	3200
1633	286	1658	960	1683	29	1708	416
1634	*	1659	208	1684	30	1709	68
1635	*	1660	514	1685	27	1710	116
1636	298	1661	598	1686	30	1711	1163
1637	213	1662	772	1687	29	1712	2736
1638	236	1663	*	1688	30	1713	2712
1639	174	1664	239	1689	11	1714	3500
1640	191	1665	959	1690	36	1715	873
1641	307	1666	*	1691	31	1716	2219
1642	210	1667	1241	1692	31	1717	1526
1643	143	1668	265	1693	52	1718	1282
1644	158	1669	697	1694	32	1719	1800
1645	200	1670	398	1695	39	1720	1286
1646	151	1671	*	1696	*	1721	2656
		1672	250	1697	29	1722	3285
		1647	941				

* missing value

Table 10. Proximal tarsometatarsal bone volumes(%)

control ♀'s			expt ♀'s			control ♂'s			expt ♂'s		
bird no.	cancell-ous bone	medull-ary bone	bird no.	cancell-ous bone	medull-ary bone	bird no.	cancell-ous bone	medull-ary bone	bird no.	cancell-ous bone	medull-ary bone
1623	18.56	0.00	1648	*	*	1673	23.30	0.00	1698	19.25	0.00
1624	17.86	0.00	1649	19.31	0.00	1674	26.79	0.00	1699	13.22	0.00
1625	16.34	0.00	1650	17.42	0.00	1675	*	*	1700	20.78	0.89
1626	*	*	1651	15.41	0.00	1676	25.94	0.00	1701	23.41	0.11
1627	18.67	0.00	1652	21.50	0.00	1677	23.08	0.00	1702	12.11	0.00
1628	15.11	0.00	1653	*	*	1678	*	*	1703	13.29	4.29
1629	*	*	1654	*	*	1679	23.61	0.00	1704	*	*
1630	*	*	1655	*	*	1680	24.78	0.00	1705	16.61	0.00
1631	15.28	8.11	1656	*	*	1681	*	*	1706	14.11	0.00
1632	17.78	7.53	1657	21.86	0.00	1682	25.72	0.00	1707	9.77	0.00
1633	13.14	0.00	1658	16.89	0.00	1683	*	*	1708	15.92	3.25
1634	13.58	6.11	1659	18.92	0.00	1684	22.71	0.00	1709	21.56	0.00
1635	14.08	0.92	1660	18.19	0.00	1685	19.61	0.00	1710	14.69	0.00
1636	18.30	0.00	1661	19.21	0.00	1686	24.03	0.00	1711	10.47	2.86
1637	13.78	0.83	1662	20.75	0.00	1687	23.72	0.00	1712	20.63	0.00
1638	*	*	1663	22.25	0.00	1688	27.36	0.00	1713	14.44	2.50
1639	17.09	0.00	1664	26.58	0.00	1689	21.92	0.00	1714	*	*
1640	20.03	0.00	1665	20.55	0.00	1690	*	*	1715	13.04	1.17
1641	13.94	3.89	1666	18.92	0.00	1691	23.78	0.00	1716	12.14	2.69
1642	18.50	6.02	1667	26.25	0.00	1692	21.22	0.00	1717	10.94	0.00
1643	16.00	2.25	1668	*	*	1693	22.89	0.00	1718	10.64	0.28
1644	13.25	2.08	1669	18.62	0.00	1694	28.20	0.00	1719	10.67	7.25
1645	14.33	0.00	1670	18.36	0.00	1695	19.00	0.00	1720	*	*
1646	13.94	10.61	1671	20.00	0.00	1696	28.17	0.00	1721	*	*
			1672	19.75	0.00	1697	23.72	0.00	1722	*	*
			1647	22.78	0.00						

Table 11. Tarsometatarsal cortical thickness (mm)

control ♀'s		exp ♀'s		control ♂'s		exp ♂'s	
bird no.	thickness (mm)	bird no.	thickness (mm)	bird no.	thickness (mm)	bird no.	thickness (mm)
1623	0.53	1648	0.51	1673	0.60	1698	0.46
1624	0.42	1649	0.56	1674	0.58	1699	0.48
1625	0.41	1650	0.40	1675	0.57	1700	0.55
1626	0.44	1651	0.56	1676	0.47	1701	0.43
1627	0.51	1652	0.42	1677	0.46	1702	0.42

Table 12. Mean Values(± S.D.)

	Control ♀	Expt. ♀	Control ♂	Expt. ♂
Body Wt (kg)	1.83 (± 0.19)	1.84 NS (± 0.12)	2.68 (± 0.20)	2.72 NS (± 0.38)
Plasma Ca (pg/ml)	3.73 (± 2.07)	1.37 *** (± 0.73)	0.93 (± 0.21)	5.47 *** (± 3.83)
Plasma Oest (pg/ml)	202.1 (± 70.9)	768.5 *** (± 415.4)	28.79 (± 9.62)	1377 *** (± 1192)
PTM TBV (%)	15.98 (± 2.20)	20.18 *** (± 2.81)	23.98 (± 2.54)	14.88 *** (± 4.17)
PTM MBV (%)	2.42 (± 3.39)	0.00 ***	0.00	1.26 *** (± 1.97)
cortical thickness (mm)	0.46 (± 0.05)	0.49 NS (± 0.08)	0.54 (± 0.07)	0.47 * (± 0.05)

NS	not significant
*	$p < 0.05$
**	$p < 0.01$
***	$p < 0.001$

The first aim of the present experiment was to investigate the effects of oestrogen-induced medullary bone formation on cancellous and cortical bone of male fowl. The effects of oestrogen administration on plasma calcium and oestradiol were monitored

Plasma oestradiol values in oestrogen-treated male birds were in all cases greater than controls they were extraordinarily variable, in some cases being in excess of 100 times greater than the control mean. Plasma calcium levels were increased by a factor of 5 in the oestrogen-treated group compared with controls. The effect of oestrogen treatment on this parameter was also highly variable, 42% of the birds sampled having plasma calcium values similar to controls.There was no relationship between plasma calcium and oestradiol values; some of the birds with very high plasma oestradiol values had low plasma calcium values and vice versa.

In a study of oestrogen-induced medullary bone formation in male pigeons, the hypercalcaemic response to oestrogen was also variable (Pfeiffer & Gardner, 1938), though not as markedly as in the birds of the present study. Laying fowl are known to differ in their hypercalcaemic response to oestrogen; plasma calcium is elevated to much higher levels and is accompanied by hyperlipaemia (Landauer et al, 1941). This hyperlipaemia was grossly apparent in the present study, making accurate pipetting of plasma difficult and possibly contributing to the large degree of variation in plasma parameters in this group. In the case of oestradiol, high levels of plasma lipid can interfere with the extraction procedure in the assay, and may have contributed to the variation (H.Mc Cormack, personal communication). However the present study did not enable conclusions to be drawn regarding the relationships betwen plasma calcium, oestradiol and bone parameters in individual birds; further study would be necessary to determine these relationships.

The hypercalcaemic response of birds and other submammalian vertebrates to oestrogen is well documented (Dacke, 1993). Alligators, lizards, turtles, and snakes all respond to oestrogen by increasing plasma calcium levels (Elsey & Wink, 1986). Despite the fact that many of these reptiles lay eggs with calcareous shells, none have been shown to develop

medullary bone. In some lizards, the endolymphatic sacs of the female become engorged with calcium carbonate deposits at the time of reproduction, and shortly before oviposition these stores are depleted (Ruth, 1918). The bones of female turtles have been shown to decrease sharply in density at the time of egg-shell calcification (Edgren, 1960). More recent studies of alligators has indicated that they also resorb structural bone during egg-shell formation (Elsey & Wink, 1985), but show no cortical bone density changes in response to oestrogen administration (Elsey & Wink, 1986).

All egg-laying birds examined to date have been found to have medullary bone (Simkiss,1961), and it is considered to be an evolutionary adaptation to the calcium demands of egg-shell formation (Miller, 1992). The logical conclusion is that medullary bone, under normal circumstances, acts as a temporary, labile calcium reservoir, thus protecting and preserving skeletal integrity. This is probably true of birds which produce a clutch of eggs over a short period of time then incubate them; medullary bone serving its purpose during shell formation then being rapidly resorbed. Even if the process of medullary bone modelling results in a temporary decrease in cancellous bone volume, this negative effect will firstly be compensated for by medullary bone's protective effect during remodelling, and secondly, will be promptly repaired on completion of the clutch. Commercial egg-laying hens, in contrast, are far removed from this natural situation. They produce eggs almost continuously for a year, and their medullary bone should not be considered a temporary tissue because it is present (in increasing volume) for most of the bird's life. The subsequent prolonged period of high bone turnover to meet calcium demand results in deleterious effects on structural bone, causing osteoporosis and fractures.

The initial cancellous bone loss associated with medullary bone modelling in pullets in Chapter 2. had not been described previously. However, structural bone loss associated with oestrogen-induced medullary bone formation in males has been described previously, in ducks and pigeons as well as chickens. In male pigeons, there is evidence that cortical thinning and resorption occurs during oestrogen-induced medullary bone formation (Pfeiffer & Gardner, 1938). In oestrogen-treated ducks, a loss of cancellous bone trabeculae was

described in the femoral diaphysis (Landauer & Zondek, 1944). They also indicated that the most outstanding histological feature of the long bones of adult cockerels after low doses (0.17-0.23g/day for 4 weeks) of oestrogen was destruction and resorption of bone. Thinning of cortical bone and increased intracortical remodelling and osteoclast numbers accompanied increased osteoblast numbers and limited medullary bone formation on endocortical and endosteal surfaces. At higher doses, these signs of bone destruction became less prominent, medullary bone formation being the most obvious change. More recently, Turner et al (1993) reported that a reduction in cortical bone area accompanied the hypercalcaemia and medullary bone formation induced by oestrogen administration to male Japanese quail.

In the present experiment, it was found that oestrogen treatment of cockerels resulted in highly significant decreases in cancellous bone volume ($p< 0.001$), accompanied by medullary bone modelling. There was no significant effect on cortical thickness. It is possible that such structural bone loss is a normal, but variable, prelude to medullary bone modelling in birds. It would be necessary to compare the responses to oestrogen of different species of male birds to confirm this, and the variable responses of the different bone envelopes would provide further interesting areas of study. As discussed previously, such a temporary structural bone loss may be a small cost of providing a larger, more labile source of mineral for shell-production. It may however, have serious consequences for birds selected for prolonged periods of egg production, such as the modern commercial layer.

The dose related cortical bone loss reported in oestrogen-treated cockerels (Landauer & Zondek, 1944) was also relevant to the present study. The initial cancellous bone loss described in Experiment 1. occurred before plasma oestradiol peaked and before maximal secretion of oestrogen from the thecal cells. However, the degree of cancellous bone loss in the birds in the present experiment did not correlate with plasma oestradiol values. However, because of the numbers of birds used in this experiment, it was not possible to determine such a relationship.The effects of varying doses or levels of circulating oestrogen on bone parameters would be an interesting area for further study.

In oestrogen-treated birds with well-developed medullary bone, either of two distinct types were seen. The first was the spicule type medullary bone found naturally in females and all other descriptions of oestrogen-induced medullary bone formation in male birds (Pfeiffer & Gardner,1938; Landauer & Zondek,1944; Miller & Bowman, 1981; Bowman & Miller, 1986; Oshashi et al 1991; Turner et al, 1993). The second type was formed in narrow bands, parallel to the cancellous bone surface. There is no apparent reference to this type of medullary bone formation in the literature. In the most recent studies of those cited above, oestrogen administration was short term and designed to examine the cellular changes occurring during the "endosteal reaction". In the present study, oestrogen was administered for a 2 week period, during which time the remodelling pressures of the egg-laying cycle were obviously absent. However this does not really explain the appearance of the band-type medullary bone because it was present in only some of the birds, and in the earliest work cited above, the duration of oestrogen treatment was in excess of 4 weeks.

The second aim of the experiment was to determine whether the structural bone loss described in laying hens in Chapter 1. could be affected by the administration of an anti-oestrogenic substance previously shown to prevent medullary bone formation in male oestrogen-treated quail (Oshashi et al, 1988; Williams et al, 1991)l. Tamoxifen suppressed development of the ovarian follicles beyond 12mm in diameter, and despite causing massive increases in plasma oestradiol levels, also prevented the characteristic avian hypercalcaemic response to oestrogen. These responses of the reproductive system and plasma parameters to tamoxifen are essentially similar to those described by Jaccoby et al(1992). In their study, it was found that tamoxifen at low doses advanced the onset of sexual maturity, while it was prevented at high doses. They concluded that tamoxifen enhanced ovarian steroidogenesis, thus increasing plasma oestrogen concentrations. However, at doses high enough to compete with the elevated oestrogen in circulation, tamoxifen decreased oestrogen effectiveness in peripheral target tissues.

The histomorphometry and histology results showed that tamoxifen prevented medullary bone formation in all of the treated hens, and also prevented the cancellous bone loss which

accompanies medullary bone modelling. It was interesting that although the bone lining cells appeared activated, and osteoclasts were observed, none were closely adhered to the bone surface and there was no reduction in cancellous bone volume to indicate resorption had taken place. It is possible that in this experiment, the rise in plasma oestradiol initially activated the bone lining cells but that other factors subsequently involved in bringing about resorption were inhibited. The resorption of bone and subsequent hypercalcaemia normally induced by oestrogen were prevented by tamoxifen, but the mechanisms involved are beyond the scope of the present experiment and require further investigation.

In birds, as in mammals, oestrogen clearly has a major influence on osteogenesis. However, there are obvious differences in the effects in different species. In mammals, oestrogen is known to inhibit resorption and stimulate formation (Takano-Yamamoto & Rodan, 1990). Oestrogen withdrawal can result in increased activation of bone remodelling units, with incomplete filling of resorption lacunae and eventual decreases in bone mass (Parfitt, 1988; Steiniche et al, 1989; Eriksen et al, 1994). In birds, oestrogen results in massively increased bone turnover and the formation of medullary bone. This oestrogen-dependent bone formation is linked to calcium metabolism, and can function as a mineral reservoir for shell formation, without damaging skeletal integrity. This is a great advantage for a species which has a capacity for flight and therefore must have been under considerable evolutionary pressure to maintain a light but strong skeleton (Miller, 1992). In contrast, reptiles, perhaps because of their greater bone mass, do not form medullary bone in response to oestrogen, despite laying calcareous eggs (Dacke, 1979). As a consequence, alligators and turtles, for example, experience structural bone loss during egg-shell calcification (Edgren, 1960; Elsey & Wink, 1985).

The results of this experiment confirm that oestrogen is necessary for medullary bone formation. Additionally, they indicate that oestrogen also has a role in resorption; in oestrogen-treated males, medullary bone formation was accompanied by cancellous bone loss, while in tamoxifen-treated females, the prevention of medullary bone formation was accompanied by the maintenance of cancellous bone. This effect of oestrogen on resorption

may be indirect, because although oestrogen receptors have been detected immunohistochemically in bone lining cells and osteoblasts in quail, their presence has not been detected in osteoclasts (Oshashi et al, 1991; Turner et al, 1993). Further work is necessary to determine the mechanisms by which oestrogen modifies bone metabolism in birds.

THE EFFECTS OF THE BISPHOSPHONATE ALENDRONATE ON THE STRUCTURAL BONE LOSS ASSOCIATED WITH MEDULLARY BONE MODELLING AND REMODELLING.

Results from the previous experiments in this study indicate that in laying strain domestic fowl, structural bone loss, and the subsequent development of osteoporosis, is associated with medullary bone modelling and remodelling. The onset of ovarian follicular development marks the start of structural bone loss; therefore peak bone mass in the laying hen is achieved approximately 10 days prior to the production of its first egg. Thereafter, the presence of fluorochrome bone labels indicate that medullary bone is produced throughout the egg laying cycle, while the absence of label in structural bone suggests no structural bone formation occurs during the same period. However, the significant decreases in structural bone volume which occur during medullary bone modelling and remodelling are indicative of continued resorption.

The osteoporosis typical of post-menopausal women is associated with the loss of oestrogen and the most effective treatment involves oestrogen replacement (Lindsay & Cosman, 1992). The reverse is true of laying hens, and indeed tamoxifen treatment prevents structural bone loss. However, prevention of follicular development in pullets in a commercial setting is obviously senseless and although delaying the onset of lay would probably also have a positive effect on peak bone mass it is unlikely to be popular with egg producers for economic reasons. Attempts to increase structural bone formation after point of lay would probably be futile because at this time only medullary bone is being formed. Clearly, any attempt to prevent the severe osteoporosis typical of commercial egg-laying hens should focus on either preventing structural bone resorption during medullary bone modelling and remodelling, or maximising peak bone mass, or a combination of both.

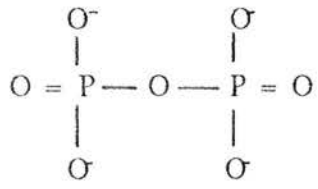
The bisphosphonates are a group of drugs developed in the past two decades for use in various disorders of bone and calcium metabolism. They have similar physicochemical properties to pyrophosphate, which occurs naturally in plasma and urine. They bind avidly to calcium phosphate, impairing crystal formation and dissolution (Hughes et al, 1991; Fleisch,1993). Pyrophosphate, however, has no effect on bone resorption because it is rapidly

hydrolysed when administered orally and to sites of resorption. In contrast, the bisphosphonates are completely resistant to enzymatic hydrolysis. They are synthetic analogues of pyrophosphate and are characterised by two C-P bonds, replacing the O-P bonds of pyrophosphate. The most skeletally active forms have both the bonds on a single carbon atom. This P-C-P structure permits a great number of possible variations; the two lateral chains on the carbon atom can be changed or the phosphate groups can be esterified, giving rise to the different bisphosphonates known as etidronate, clodronate, pamidronate, alendronate, tiludronate, and CGP42'446 (Figure 35).

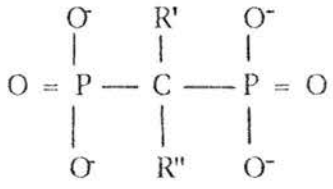
The main biological effect of the bisphosphonates is to inhibit bone resorption, but the "coupling" of resorption and formation eventually results in a reduction in bone turnover. Although the exact mode of action of the bisphosphonates in preventing resorption is unknown, it is generally thought that they are deposited in bone because of their strong affinity for bone mineral, and that they then inhibit osteoclast action on bisphosphonate-containing bone (Papapoulos, 1992; Fleisch, 1993). Individual bisphosphonates vary in their potency to inhibit bone resorption; in rats, alendronate is 10-100 times more potent than etidronate, while CGP 42' 446 has greater than 10 times the potency of alendronate. Although all skeletally effective bisphosphonates have a P-C-P structure, (which is necessary for mineral binding), the intensity of the antiresorptive effect is dependent on the characteristics of the side chain. The antiresorptive effect increases with chain length up to a maximum of four carbons, and also with the addition of a hydroxyl group to the carbon atom at position 1, or a nitrogen atom.

The efficacy of bisphosphonates in the treatment of conditions characterised by excessive osteoclastic bone resorption has been established in man and other mammals. For example, malignancy-associated hypercalcaemia and Paget's disease have been successfully treated with bisphosphonates (Papapoulos et al, 1992; Fleisch, 1993).

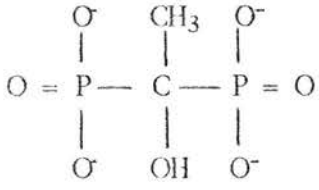
The treatment of post-menopausal osteoporosis with bisphosphonates is more complicated, firstly because the former is rarely associated with increased resorption, and secondly because



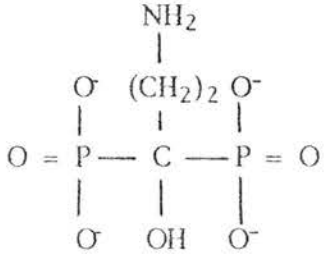
Pyrophosphate



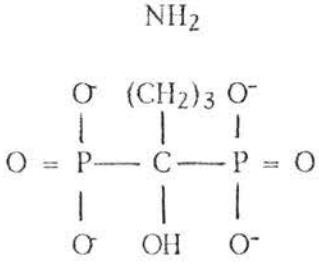
Geminal Bisphosphonate



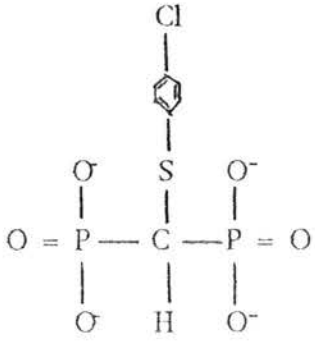
Etidronate



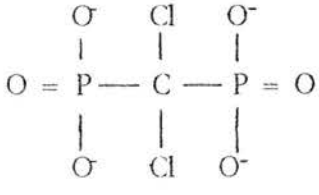
Pamidronate



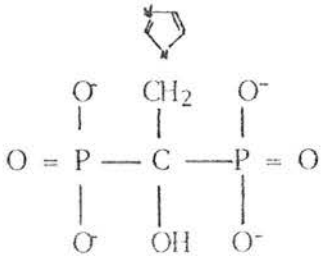
Alendronate



Tiludronate



Clodronate



CGP 42' 446

Figure : The chemical structure of pyrophosphate and various bisphosphonates

of the subsequent detrimental effects of bisphosphonates on bone formation rates (Papapoulos et al, 1992). In the 1970's, etidronate was used in high doses to treat osteoporosis but was found to interfere with the mineralisation of new bone (Heaney & Saville, 1976). It was later discovered that trabecular bone volume could be increased by the use of etidronate intermittently (Anderson et al, 1984) as part of a coherence or ADFR therapy (Frost, 1981). Many studies have since been carried out using this approach, with conflicting results (Pacifici et al, 1988; Hodsman,1989; Storm et al, 1990; Watts et al, 1990). The data generated by these studies suggests that intermittent administration of etidronate may have a favourable effect on new spinal fractures in patients with established post-menopausal osteoporosis but they are not conclusive.

Evidence from studies carried out over the last ten years on the newer bisphosphonates is more positive. Uninterrupted administration of low doses only mildly suppresses bone turnover and modulates bone metabolism in a favourable direction (Papapoulos, 1994). Further controlled trials are required to confirm this.

Another more recent application of bisphosphonates is the prevention of post-menopausal bone loss. Tiludronate administered for 6 months to post-menopausal women arrested bone loss and this effect was sustained 6 months after treatment (Reginster et al, 1989). Further study is however required and bisphosphonate treatment would have to compare favourably with the established and relatively safe oestrogen therapy before being used widely.

Alendronate is a highly potent geminal bisphosphonate which inhibits osteoclastic bone resorption but does not impair bone mineralisation. It is an inhibitor of resorption in vitro (Sato & Grasser,1990), in experimental animals (Schenk et al,1986; Thompson et al, 1990), and in patients with Paget's disease (Pedrazzoni et al, 1989; O'Doherty et al, 1990; Burssens et al, 1990; Seedor et al,1991).

In experiments investigating localisation of labelled alendronate in rat bones (Sato et al, 1991), 72% of osteoclastic surface, 2% of bone forming surface, and 13% of other surfaces

were found to be densely labelled 1 day after subcutaneous injection of 0.4mg/kg. Six days after injection, the labelled alendronate was located 600-1000μm from the bone surface, indicating normal bone formation onto treated bone. In the same study, alendronate bound to bone particles (at a concentration of $1.3x10^{-3}$fmol/mm^2 bone surface), inhibited bone resorption by isolated chicken osteoclasts. This bone surface concentration produced a concentration of 0.1-1mmol in the resorption space, causing increased leakiness to calcium in 20-30% of osteoclasts. This may stop osteoclast activity before damaging the cells. Inhibition of activity would then prevent further acidification, reduce bisphosphonate concentration in the resorption space and restore membrane integrity. Osteoclast numbers were not reduced by alendronate treatment, suggesting these events did not lead to osteoclast death. The authors proposed that the mechanism of action of alendronate is:

1) it binds preferentially to exposed hydroxyapatite surfaces prepared for bone resorption

2) it is locally released at high concentrations by osteoclastic acidification, and

3) it increases the leakiness of the ruffled border to ions, preventing resorption and maintenance of the ruffled border.

One approach to preventing osteoporosis in laying hens would be to prevent the structural bone resorption associated with medullary bone modelling and remodelling. The bisphosphonates are powerful inhibitors of osteoclastic action and may therefore be useful, but no information regarding their efficacy in birds is available. The final experiments in this study aim to investigate the effects of the bisphosphonate alendronate on the structural bone loss associated with medullary bone modelling and remodelling.

Histomorphometric analysis of bone volumes in samples from birds given various doses of the alendronate during the laying cycle will be carried out. In the early stages of ovarian follicular development, a period of intense osteoclastic resorption results in structural bone loss prior to medullary bone development. The effects on bone volumes of a low dose of alendronate administered to pullets around the start of follicular development will also be investigated. The latter investigation will also serve to confirm the earlier findings of the study.

Animals

Experiment 1.

20 mid-lay, 39 week-old Hisex hens (Ross Poultry) were housed in individual cages and fed water and a standard layer ration *ad libitum*. They were randomly divided into four groups of five individuals. Every second day for 2 weeks three of the groups of hens received (by subcutaneous injection) 1ml/kg sterile water containing alendronate (Merk, Sharp & Dohme) at concentrations of either 1.0, 0.10, or 0.01mg/ml . The fourth group served as controls and received sterile water alone. The birds were sacrificed 15 days after treatment.

Experiment 2.

20 sixteen week old Hisex pullets were housed in individual cages and fed water and a standard layer ration *ad libitum* . They were randomly divided into two groups of ten. One group was injected as described in Experiment 1. with 0.01 mg/kg alendronate in sterile water twice weekly, and the other control group with sterile water alone. Each bird was killed after laying a single egg.

Experiment 3.

94 female day old chicks (Hisex, Ross Poultry) were housed in brooders until 14 weeks of age, then transferred to individual cages . They were fed water and standard layer rations *ad libitum*. At 14 weeks of age they were divided into two groups and treated as follows; alendronate-treated group (n=48) received 6 x 0.01mg/kg alendronate in sterile water, injected subcutaneously over the superficial pectoral muscle over a two week period, and controls (n=46) received, similarly, sterile water alone. 10 birds from each group received intravenously at the time of the first and last injections 25mg/kg fluorescein complexone (BDH) and 20mg/kg oxytetracycline (Engemycin,Mycofarm UK Ltd) respectively. Half of each group were sacrificed when they had layed a single egg (experimental point-of- lay [exp POL], and control point-of-lay [control POL] groups). The remaining birds were sacrificed at 36 weeks of age after approximately 20 weeks of egg-production (experimental mid-lay [exp ML] , and control mid-lay [control ML] groups). Five birds from each of these 2 groups

received a third fluorochrome bone label, xylenol orange (90mg/kg) 3 days prior to sacrifice. Egg production was recorded for the entire duration of the experiment.

Bone samples were collected and processed for decalcified and undecalcified sections, and histomorphometric measurements carried out and analysed as described in the previous experiments of this study. (Chapter 2)

EXPERIMENT 1.

Gross pathological examination revealed some calcification of the soft tissues and oedema in the subcutaneous tissues. These affected only the birds receiving the highest dose of alendronate and were restricted to the site of injection. The reproductive tract of these birds showed no evidence of follicular activity, and egg records indicated they stopped laying eggs after 4 days. All the other birds appeared normal, with normal follicular heirarchies. There was no significant difference in the number of eggs layed between the groups receiving the two lower doses of alendronate and the controls.

Histomorphometry

Bone volumes for each bird are shown in Table 13, with mean values for each group. There was no significant difference in cancellous bone volume between the control group and any of the alendronate-treated groups. Medullary bone volume was significantly lower ($p<0.05$) in the 1.0mg/kg group than in the control group, but there were no other significant differences in medullary bone volume.

Histology

Decalcified sections indicated that all the birds were osteoporotic, having slender metaphyseal cancellous bone trabeculae. Undecalcified sections showed both the cancellous and medullary bone to be normally mineralised in all but two birds. In one bird from the 1.0mg/kg group and one bird from the 0.1mg/kg group, there were thickened seams of unmineralised matrix in the medullary bone, these being indicative of osteomalacia.

EXPERIMENT 2.

The control birds and alendronate-treated birds appeared in normal health during and at the end of the experiment. There was no significant difference in the age at which the control and alendronate-treated birds layed their first egg, and the reproductive tract showed a normal

follicular hierarchy in all the birds.

Histology

Examination of decalcified sections suggested that the cancellous bone of alendronate-treated birds was thicker and better connected than in controls. Medullary bone was present in typical quantities for point of lay hens and appeared similar in both groups. Undecalcified sections demonstrated normally mineralised cancellous and medullary bone with no evidence of osteomalacia in either group.

Histomorphometry

Cancellous and medullary bone volumes of the proximal tarsometatarsus of each bird are shown in Table 14, and also the group means and standard deviations. Cancellous bone volume was significantly higher ($p<0.05$) in alendronate-treated birds than in controls. Medullary bone volume was lower in the alendronate-treated group than in controls but the difference was not significant.

Table 13. Proximal tarsometatarsal bone volumes in point of lay birds receiving 0.01mg/kg alendronate at 16 weeks of age

CONTROL		EXPERIMENTAL	
cancellous bone volume (%)	medullary bone volume (%)	cancellous bone volume (%)	medullary bone volume (%)
24.35	4.27	19.13	3.10
17.25	3.98	18.99	1.09
13.27	1.43	21.43	0.87
*	*	14.63	1.59
17.10	3.05	20.43	3.40
15.04	2.16	21.56	2.95
13.92	3.11	27.35	4.90
14.58	4.08	25.86	2.71
17.56	1.40	26.67	0.93
17.20	4.90	17.54	2.49
χ (± S.D.)	χ (± S.D.)	χ (± S.D.)	χ (± S.D.)
16.69 (+ 3.29)	3.15 (+ 1.27)	21.36 * (+ 4.16)	2.43^{NS} (+ 1.29)

NS- not significant

* - $p<0.05$

Table 14. Bone volumes of hens receiving alendronate mid-lay

control		0.01mg /kg		0.10mg /kg		1.0 mg /kg	
CBV(%)	MBV(%)	CBV(%)	MBV(%)	CBV(%)	MBV(%	CBV(%)	MBV(%)
4.62	12.36	14.04	13.25	10.53	5.81	10.51	3.83
6.58	12.00	9.96	6.67	8.38	14.06	9.94	4.51
10.50	14.88	12.35	8.41	13.14	9.51	12.66	11.79
8.97	13.06	9.94	4.51	9.27	10.41	10.29	13.04
16.87	10.00	13.59	7.20	9.87	8.00	8.11	7.05
χ (± SD)		χ (± SD)		χ (± SD)		χ (± SD)	
9.51 (±4.69)	12.46 (±1.77)	11.98^{NS} (±1.95)	8.01^{NS} (±3.25)	10.24^{NS} (±1.80)	9.56^{NS} (±3.06)	10.30^{NS} (±1.62)	8.04 * (±4.19)

NS - not significant
* - $p<0.05$

EXPERIMENT 3

The age at which each bird layed its first egg is shown in Table 15, and the mean values for each group in Table 20. There was no significant difference in the age at which lay commenced between the alendronate-treated and control groups.

Egg production for each of the birds killed at 36 weeks is shown in Table 16, and mean values for the two groups in Table 20. There was no significant difference in egg production between the alendronate-treated and control groups.

Histology

Controls

At point of lay, the control birds showed evidence of diminished cancellous bone, the trabeculae appearing thin and discontinuous. The cancellous and endocortical surfaces were covered in spicules of medullary bone which were lined with an abundance of osteoblasts and osteoclasts (Figure 36). The cortices were thick and showed no sign of remodelling. In undecalcified sections, it was observed that the bone was normally mineralised. 12μm sections examined under transmitted ultra violet light demonstrated the presence of labels either both in cancellous bone, or the first label in cancellous bone and the second in medullary bone (Figure 37), depending on the age at which the bird commenced lay i.e. the latter came into lay sooner than the former.

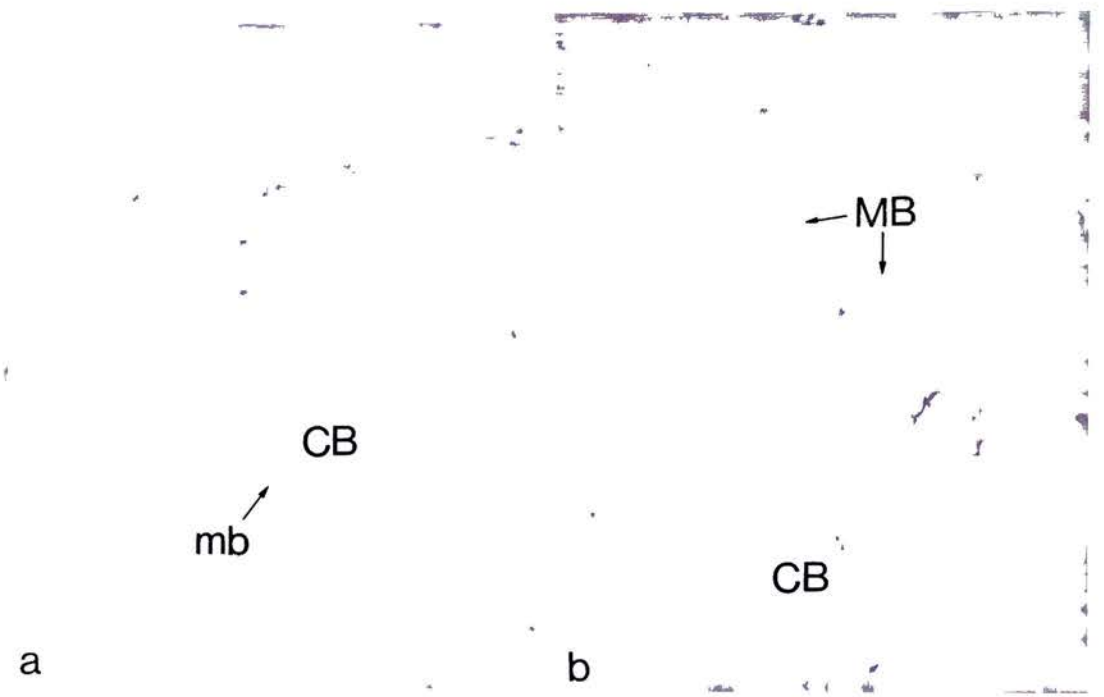
At mid-lay (36 weeks), decalcified bone sections had greater quantities of medullary bone than at point-of-lay. Medullary bone spicules were present throughout the bone and not confined to cancellous bone trabeculae as in the point of lay group (Figure 36). Massive osteoclasts were present around these medullary bone spicules (Figure 36), as were numerous osteoblasts. The cortices had become extensively trabecularised and porous, the resorption cavities being lined with medullary bone. Undecalcified sections indicated that the cancellous and medullary bone were mineralised normally (Figure 38). The fluorochrome labelling administered during follicular development persisted in places in the cancellous bone, and the

third label, which was administered during lay was abundant in medulary bone spicules (Figure 37). No other bone was labelled with the third label.

Alendronate-treated

At point of lay, the birds in the alendronate-treated group appeared to have more cancellous bone than controls. The trabeculae were thicker and they seemed to anastomose more frequently. Otherwise they were similar to the controls. In undecalcified sections the bone was normally mineralised. The location of the fluorochrome labels provided some information regarding the type of bone being mineralised at the time of alendronate administration. Of the five birds receiving bone labels, three were mineralising cancellous bone both at the time of the first and last alendronate injection, while the remaining two birds were mineralising cancellous bone at the first and medullary bone at the time of the last injection.

The birds in the mid-lay alendronate-treated group were similar to the controls in the amount and distribution of medullary bone, the appearance of the cortices and the persistence of the first and second labels in the cancellous bone. However, there was more cancellous bone, and in undecalcified sections it was observed that the medullary bone had thicker osteoid borders than in controls (Figure 39).



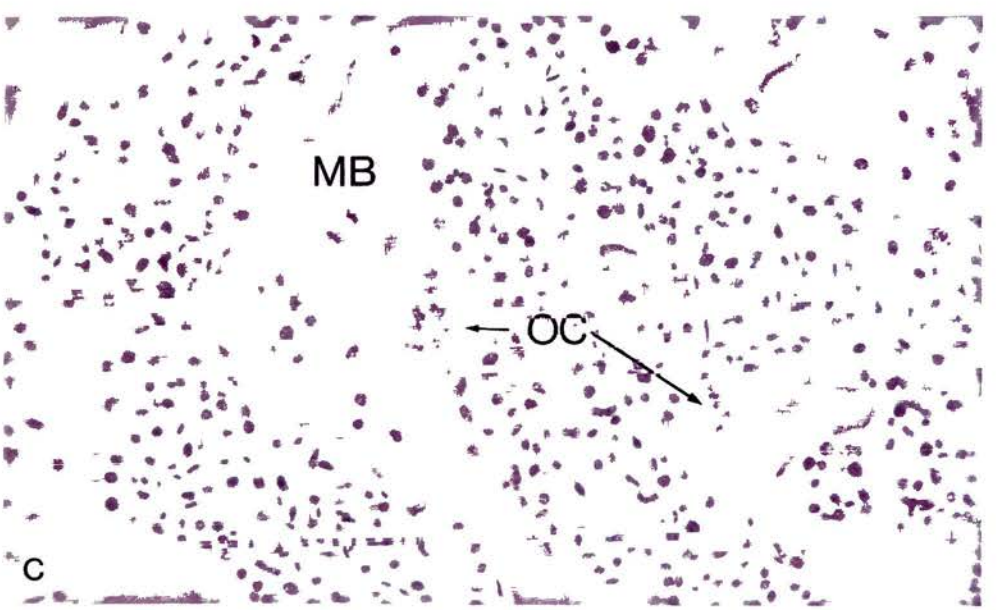


Figure 36. Decalcified sections from control birds, stained H&E; a) point of lay (x95) b) mid-lay (x95) and c) mid-lay (x470). MB-medullary bone CB-cancellous bone OC-osteoclast OB-osteoblast

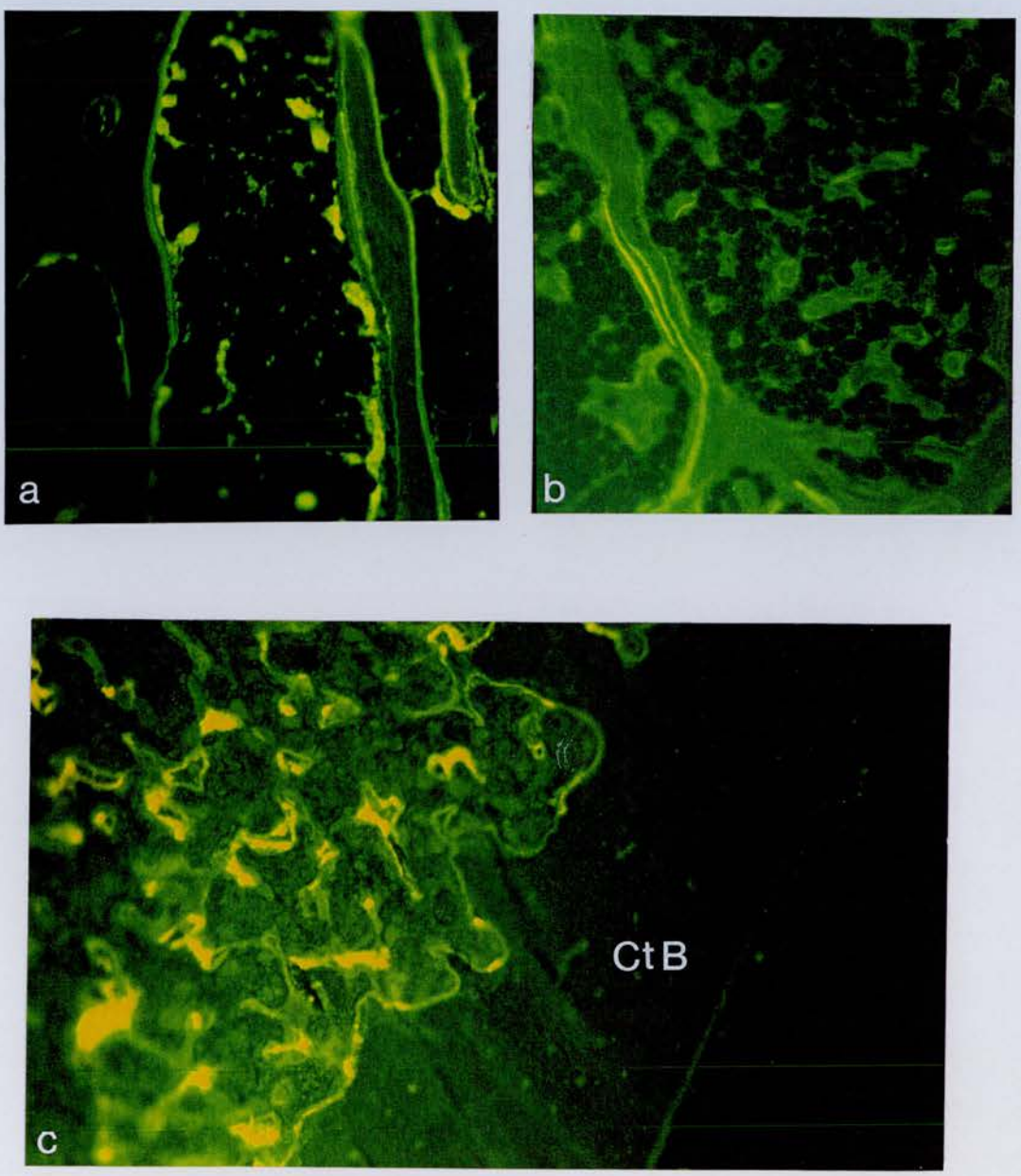


Figure 37. Undecalcified unstained sections labelled with oxytetracycline (OTC) and fluorescein complexone (FC), or xylenol orange (XO); a) control point of lay bird (x 95) b)control mid-lay bird (x 95) and c) control mid lay (x240) Cb-cancellous bone MB-medullary bone CtB- cortical bone

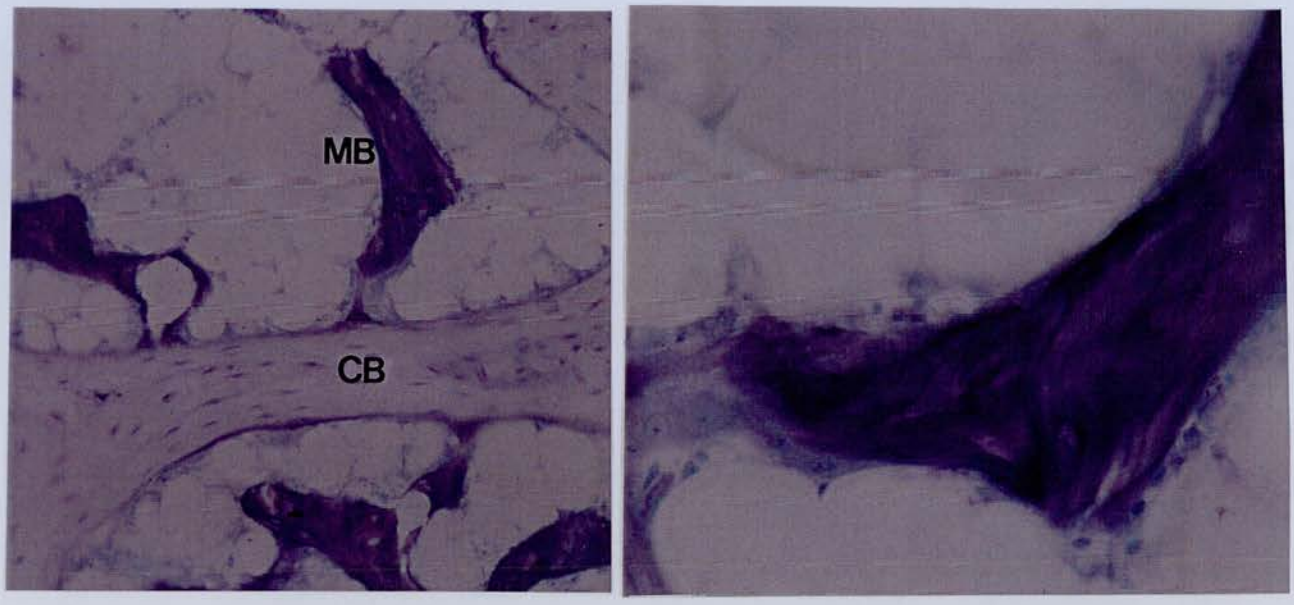


Figure 38 Undecalcified toluidine blue stained sections from control birds at mid-lay. Medullary bone trabeculae are well mineralised MB-medullary bone CB-cancellous bone (x 240 left and x 470 right)

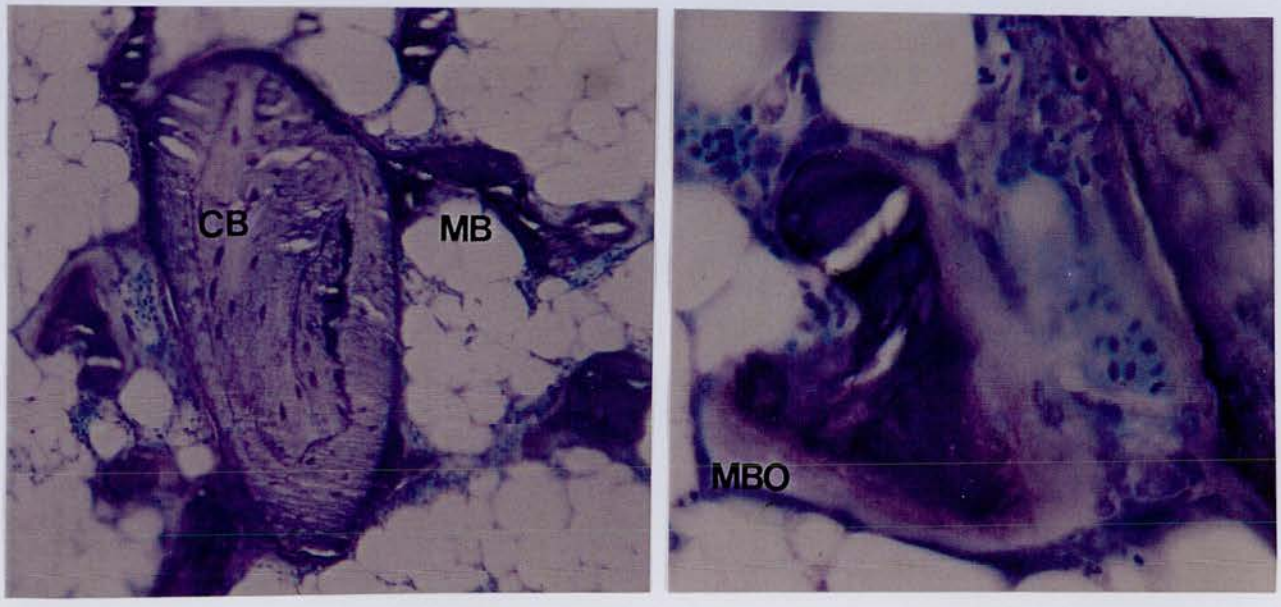


Figure 39 Undecalcified toluidine blue stained sections from alendronate birds at mid-lay. Medullary bone trabeculae less well mineralised than controls MB-medullary bone MBO-medullary bone osteoid CB-cancellous bone (x240, left and x 470, right)

HISTOMORPHOMETRY

Tarsometatarsal cancellous bone volume for each bird is shown in Table 17, medulary bone volume in Table 18, diaphyseal cortical thickness in Table 19 and mean vaalues for the four groups in Table 20.

At point of lay, cancellous bone volume was significantly higher ($p<0.01$) in alendronate-treated birds than control birds. There was no significant difference in medullary bone volumes or cortical thickness between the two groups.

At mid-lay, caancellous bone volume remained significantly higher ($p<0.001$) in the alendronate-treated group than in the controls, and medullary bone volume was significantly lower ($p<0.05$). There was no significant difference in medullary bone volumes or cortical thickness between the two groups. Between point of lay and 36 weeks of age, cancellous bone volume decreased significantly ($p<0.001$) in both alendronate-treated and control groups aand medullaary bone volume increased significantly ($p<0.001$).

Table 15. Age at first egg (days)

control POL		exp. POL		control ML		exp. ML	
bird no.	age	bird no.	age	bird no.	age	bird no.	age
340	109	364	122	873	125	897	143
341	123	365	125	874	133	898	117
342	136	366	126	875	130	899	125
344	107	367	115	876	129	900	121
345	132	368	142	877	137	901	115
346	142	369	129	878	137	902	138
347	142	370	120	879	113	903	117
348	115	371	119	880	122	904	130
349	117	372	122	881	115	905	120
350	129	373	115	882	124	906	116
352	126	374	121	883	114	907	134
353	138	375	119	884	121	908	130
354	130	376	122	885	124	909	126
355	129	377	122	886	122	910	135
356	121	378	124	887	129	911	126
357	129	379	136	888	125	912	120
358	139	380	124	889	133	913	144
359	116	381	142	890	140	914	115
360	129	382	139	891	125	915	123
361	121	383	121	892	128	916	128
362	125	384	132	893	124		
		385	121				
		386	142				
		387	122				

Table 16. Egg production (total eggs)

control	POL	exp.	POL	control	ML	exp.	ML
bird no.		bird no.		bird no.		bird no.	
340	-	364	-	873	105	897	97
341	-	365	-	874	111	898	100
342	-	366	-	875	92	899	102
344	-	367	-	876	73	900	101
345	-	368	-	877	90	901	96
346	-	369	-	878	98	902	112
347	-	370	-	879	122	903	113
348	-	371	-	880	107	904	116
349	-	372	-	881	109	905	105
350	-	373	-	882	98	906	84
352	-	374	-	883	103	907	92
353	-	375	-	884	94	908	99
354	-	376	-	885	96	909	105
355	-	377	-	886	98	910	95
356	-	378	-	887	93	911	107
357	-	379	-	888	91	912	95
358	-	38o	-	889	109	913	110
359	-	381	-	890	98	914	95
360	-	382	-	891	84	915	102
361	-	383	-	892	73	916	100
362	-	384	-	893	48		
		385	-				
		386	-				
		387	-				

Table 17. Proximal tarsometatarsal cancellous bone volume (%)

control	POL	exp.	POL	control	ML	exp.	ML
bird no.	CBV	bird no.	CBV	bird no.	CBV	bird no.	CBV
340	11.28	364	24.49	873	6.70	897	8.25
341	13.65	365	17.94	874	10.15	898	11.62
342	13.98	366	16.14	875	9.19	899	14.55
344	10.87	367	21.44	876	12.16	900	8.03
345	14.25	368	18.08	877	6.94	901	10.78
346	18.11	369	18.97	878	9.75	902	10.36
347	14.74	370	11.93	879	11.53	903	*
348	14.71	371	10.63	880	11.43	904	7.78
349	14.33	372	16.03	881	11.25	905	13.89
350	12.53	373	17.03	882	11.67	906	15.72
352	14.64	374	*	883	12.53	907	16.31
353	15.46	375	10.58	884	9.47	908	13.28
354	19.33	376	21.79	885	8.66	909	15.96
355	12.68	377	18.56	886	10.71	910	16.00
356	15.50	378	14.55	887	10.27	911	11.05
357	15.33	379	16.75	888	4.37	912	11.55
358	16.44	380	20.27	889	7.56	913	14.47
359	13.67	381	25.78	890	10.54	914	13.72
360	7.89	382	19.97	891	6.17	915	14.89
361	12.03	383	17.01	892	11.67	916	13.50
362	12.61	384	16.03	893	13.12		
		385	9.79				
		386	21.49				
		387	19.39				

*- missing value

Table 18. Proximal tarsometatarsal medullary bone volume (%)

control (POL)		exp. (POL)		control (ML)		exp. (ML)	
bird no.	MBV	bird no.	MBV	bird no.	MBV	bird no.	MBV
340	2.19	364	3.18	873	16.89	897	11.11
341	4.75	365	1.52	874	12.43	898	12.87
342	2.88	366	5.36	875	12.17	899	6.83
344	8.88	367	2.72	876	8.14	900	8.61
345	3.58	368	0.71	877	5.14	901	6.61
346	0.47	369	4.06	878	6.58	902	11.55
347	0.61	370	2.18	879	9.03	903	*
348	2.82	371	3.67	880	13.84	904	8.50
349	5.61	372	4.19	881	9.36	905	4.67
350	2.19	373	*	882	9.25	906	6.72
352	1.72	374	7.00	883	13.34	907	5.83
353	6.33	375	0.64	884	11.69	908	12.33
354	4.25	376	7.17	885	10.72	909	3.78
355	2.68	377	5.97	886	7.95	910	3.64
356	4.00	378	2.08	887	9.92	911	8.75
357	6.16	379	0.81	888	12.25	912	10.14
358	2.08	380	2.47	889	18.06	913	3.95
359	4.33	381	5.89	890	2.96	914	10.67
360	2.72	382	4.97	891	9.11	915	8.39
361	3.22	383	5.17	892	7.03	916	7.72
362	2.74	384	4.18	893	11.31		
		385	2.90				
		386	3.10				
		387	6.19				

*- missing value

Table 19. Tarsometatarsal cortical thickness (mm)

control (POL)		exp. (POL)		control (ML)		exp. (ML)	
bird no.	thickness (mm)	bird no.	thickness (mm)	bird no.	thickness (mm)	bird no.	thickness (mm)
340	0.55	364	0.59	873	0.30	897	0.29
341	0.66	365	0.56	874	0.36	898	0.43
342	0.76	366	0.55	875	0.35	899	0.40
344	0.62	367	0.60	876	0.34	900	0.34
345	0.58	368	0.52	877	0.40	901	0.34

Table 20. Mean Values (± S.D.)

	Control POL	Expt. POL	Control ML	Expt. ML
Age 1st egg (days)	125.65 (± 9.64)	125.22 NS (± 7.84)	125.62 (± 7.93)	127.29 NS (± 9.22)
Total eggs (no.)	-	-	94.86 (± 15.93)	101.30 NS (± 7.83)
PTM cancellous bone volume(%)	14.00 (± 2.48)	17.59 ** (± 4.21)	9.80 (± 2.32)	13.23 *** (± 2.78)
PTM medullary bone volume(%)	3.53 (± 1.99)	3.74 NS (± 1.98)	10.34 (± 3.61)	8.03 * (± 2.90)
cortical thickness (%)	0.63 (±0.08)	0.56 (±0.03)	0.35 (± 0.04)	0.36 (± 0.05)

NS	not significant
*	$p < 0.05$
**	$p < 0.01$
***	$p < 0.01$

The effects of bisphosphonates generally, and of alendronate in particular, were unknown in birds, although considerable work on their effects in mammals has been carried out (Schenk et al, 1986; Pedrazzoni et al, 1989; Thompson et al , 1990; O'Doherty et al, 1990; Burssens et al , 1990; Seedor et al, 1991). The three experiments in this study aimed to investigate the effects of alendronate given at different doses and reproductive stages on bone volumes in the laying hen.

In the first of these experiments, alendronate was administered at three different doses to hens half way through their egg-laying cycle. The doses of alendronate known to prevent bone resorption in man and other mammals (Adami et al, 1986; Schenk et al 1986; Attardo-Perinello et al, 1987; Sietsema et al, 1989; Thompson et al, 1990; Bickerstaff et al, 1991; Guy et al, 1993) vary according to route of administration but are designed for longer-term administration than intended in the present study. The three doses used in this experiment were designed to determine the effects of a range of doses in the short term. Intravenous administration of bisphosphonates was not desirable, because they have been shown to form aggregates with plasma calcium. This may cause a transient hypocalcaemia, or more seriously cause renal failure if a solid phase of bisphosphonate is formed in the blood (Fleisch, 1993). This effect may be exacerbated in laying hens because of their high levels of plasma calcium. Oral administration was also considered inappropriate because of alendronate's poor (<1%) absorption from the gut in mammals, although there is no available data for birds (G.Rodan, personal communication). A subcutaneous route of administration was therefore used.

The hens receiving the highest dose (1mg/kg) in this experiment were observed to have some calcification and oedema around the site of injection. Although it is known that bisphosphonates (especially the amino derivatives), can cause local toxicity and necrosis when administered subcutaneously, bisphosphonates are used commercially to inhibit ectopic ossifications and have been shown to prevent heterotopic calcification and ossification in experimental animals (Thomas & Amstutz, 1985; Fleisch, 1993). Circulating calcium levels in laying hens are extremely high, being three times higher than in non-laying hens and

mammals (Simkiss, 1967; Dacke, 1979). It is possible that this may have contributed in some way to the formation of the type of calcium complexes known to occur when bisphosphonates are administered intravenously.

In mammals, the proposed mechanism of action of alendronate is that it binds preferentially to exposed hydroxyapatite surfaces prepared for bone resorption and that its subsequent local release by osteoclastic acidification prevents osteoclast ruffled border formation and hence resorption. Alendronate also, however, binds to 2% of bone forming surfaces and 13% of other bone surfaces (Sato et al 1991). Its preferential uptake to resorption surfaces is thought to be due to the ease with which the alendronate binds to hydroxyapatite on surfaces cleared of unmineralised matrix. Studies involving [^{3}H]Alendronate have not been carried out in birds and would be necessary to fully understand its mode of action in the laying hen. However, assuming that the distribution of uptake is similar in birds and mammals, it is likely that alendronate would bind extensively to medullary bone during the egg laying cycle because it is the site of maximal exposed hydroxyapatite surface. Medullary bone, because of its large surface area and extensive vascularization, is thought to be metabolised at least 10-15 times faster than cortical bone (Hurwitz, 1965; Simkiss, 1967), and is considered to be a very active bone remodelling system (van de Velde et al, 1984). The distribution of fluorochrome bone labels administered during the laying cycle indicates widespread mineralisation occurring solely in medullary bone, at discrete sites (Expt.1: The effects of medullary bone modelling and remodelling on the development of osteoporosis), and matrix formation is known to occur at the same time as mineralisation (Van de velde, 1984,1985).

It is unknown whether the surfaces of medullary bone are prepared for resorption in the same way as in lamellar bone. Although fine seams of unmineralised matrix are visible at high magnifications in laying hens (Wilson & Duff, 1990), and at ultrastructural level in quail (Miller, 1985) and hens (Candlish, 1971) it is unclear whether all the sufaces are covered by the morphologically distinct 'endosteal membrane' characteristic of resting surfaces in mammals. The flattened, elongated bone-lining cells typical of inactive bone surfaces in lamellar bone were not observed in medullary bone in any of the hens in this study. Candlish

(1971), described endosteal lining cells associated with medullary bone, but the photographic evidence is not conclusive. There are no references to bone lining cells in the ultrastructural study carried out by Ascenzi et al (1963); medullary bone surfaces were, however, bordered by a single layer of active osteoblasts.

Another characteristic of medullary bone which may affect the binding of alendronate is its mineralisation. Medullary bone is more heavily calcified than cortical bone, and the hydroxyapatite crystals are randomly distributed throughout the matrix, exhibiting no orientation with respect to the collagen fibres (Ascenzi et al, 1963; Taylor et al 1971). The most likely consequence of this increased mineralisation may be increased binding with bisphosphonates, but this may be offset by the unknown effect of the random hydroxyapatite crystal orientation.

The birds receiving the highest dose of alendronate stopped laying eggs 4 days after the treatment started, and their ovaries did not exhibit a follicular heirarchy. The histomorphometry data indicated that although medullary bone volume was lower in this group than in controls, it persisted at a volume of 8.04%. Medullary bone normally undergoes rapid resorption when the hen stops laying eggs, and in birds in which laying has been interrupted by a calcium deficient diet, medullary bone is completely resorbed within 10 days (Wilson & Duff, 1991). This suggests that alendronate has prevented resorption of medullary bone. The remaining treated groups also had lower medullary bone volume than the controls, but only in the group receiving the lowest dose was this statistically significant. It is probable that if alendronate binds to most of the resorption sites in medullary bone, the subsequent withdrawal of calcium for egg-shell production will be severely compromised. Osteoclasts would have to mobilise mineral from unbound surfaces, either in medullary or structural bone. The cessation of egg production in birds receiving the highest dose suggests that this was not possible and may be a reflection of alendronate binding to all bone surfaces, preventing calcium mobilisation. In the birds receiving the lower doses, egg- laying proceeded as normal, indicating that calcium for egg-shell formation was mobilised from other sources. If this was the case, it would be expected that there would be a subsequent

reduction in structural bone volume. In these animals, cancellous bone volume was insignificantly higher in the alendronate-treated groups than in controls. There was no attempt to measure cortical thickness. The large degree of variation within small groups renders a conclusion difficult to make.

Medullary bone was also observed to be osteomalacic in one bird from each of the two highest dose alendronate-treated groups. The processes of medullary bone resorption and matrix formation both occur during egg-shell formation, but at different sites. Mineralisation of this newly formed matrix occurs later, in the remaining parts of the egg-formation cycle (van de Velde et al, 1984, 1985). Osteomalacia in laying hens increases the amount of osteoid present in medullary bone, and is readily induced by a diet deficient in calcium or vitamin D (Antillon et al, 1977; Wilson & Duff, 1991). The increased amounts of unmineralised matrix seen in the two treated groups may represent a lack of available calcium to mineralise the matrix being formed at the time of alendronate administration. Alternatively, many of the bisphosphonates are known to inhibit subsequent mineralisation at the binding site when given in high doses (Fleisch, 1993). This has not previously been shown to occur with alendronate in mammals (Sato et al, 1993) but may be a possible adverse effect of higher dosages in birds.

In the second study, alendronate was administered at the lowest dose (0.01mg/kg) used in the first experiment, to pullets before medullary bone modelling. The cancellous bone surfaces were shown, in a previous experiment (Chapter 2: The effects of medullary bone modelling and remodelling on the development of osteoporosis), to undergo widespread changes during the early stages of ovarian follicular development. The bone lining cells became activated and may have been involved in preparing the bone surface for the rapid resorption which was measured in later stages of follicle maturation. Alendronate was administered twice a week from 16 weeks of age, in an attempt to provide the dose before the crucial period of follicular development, when it was known that cancellous bone formation ceased and medullary bone formation commenced. It was believed that binding of alendronate to cancellous bone may prevent its resorption during medullary bone modelling.

Histomorphometric measurement indicated that alendronate treatment resulted in significant increases in cancellous bone volume compared with controls. Medullary bone volume was lower in alendronate-treated birds but the difference was not statistically significant. The age at which the two groups layed their first egg was not significantly different.

The preferential binding of alendronate to sites of resorption, (if it occurs in birds), would probably result in extensive binding to cancellous and endocortical surfaces during the early stages of follicular development. Medullary bone formation occurred normally on the cancellous bone surfaces, indicating that alendronate treatment did not affect subsequent formation at the dose given. This dose also did not appear to influence the availability of calcium for mineralisation of medullary bone; there was no evidence of osteomalacia.

Treatment of pullets with a low dose of alendronate therefore appeared to prevent the cancellous bone loss associated with medullary bone modelling. However, it remained unanswered whether or not the effect of treatment would persist throughout medullary bone remodelling. Also, the effects of pre-lay alendronate treatment on egg-production were unknown and required investigation.

The third, more extensive and detailed experiment was carried out to address some of these issues. At point of lay, alendronate-treatment resulted in essentially similar results to the previous experiment; i.e. no significant differences in age at first egg or medullary bone volume but significantly greater cancellous bone volume. Additionally, cortical thickness was not significantly different between the treated and control groups.

Fluorochrome bone labels administered to five birds in each group at the time of the first and last alendronate injections provided information regarding their stage of follicular development. Within the alendronate-treated group killed after laying a single egg, three had both labels within the cancellous bone trabeculae, and were therefore estimated to have received treatment before medullary bone modelling. In the remaining two birds the first label was located in cancellous bone and the second in medullary bone, indicating that medullary

bone modelling was almost complete by the time of the last alendronate injection. The mean age at which these five birds layed their first egg (121.4 days), was lower than the whole group mean (125.22 days), and it is therefore probable that all the treated birds received at least the first alendronate injection in the early stages of follicular development. The effects of the exact timing of dose in relation to follicular development and binding sites would be an interesting experiment on its own but is beyond the scope of the present study. In any practical application of bisphosphonates to pullets, the animals would have to be treated at the same time, regardless of the individual's state of follicular development.

The low dose of alendronate administered to these pullets did not appear to affect mineralisation of medullary bone, the spicules of which had normal osteoid borders.

In the birds killed at 36 weeks of age, it was found that cortical thickness and egg production were not significantly different between alendronate-treated birds and controls. In contrast, medullary bone volume was significantly lower ($p<0.05$), and cancellous bone volume significantly higher ($p< 0.01$) in alendronate-treated birds. However, in both control and alendronate-treated groups, there was a significant decrease in cancellous bone volume and cortical thickness when compared with the corresponding group killed at point of lay. This was accompanied, in all cases, by a highly significant increase in medullary bone volume during the same period.

These results imply that a pre-lay dose of alendronate is effective in preventing the cancellous bone loss which is associated with medullary bone modelling, and that this advantage is maintained through medullary bone remodelling. However, alendronate did not prevent the cancellous and cortical bone loss associated with medullary bone remodelling. The bone of laying birds is subjected to a unique intensity of osteoclastic resorption, and there is evidence to suggest that avian osteoclasts are more aggressive bone resorbers than mammalian osteoclasts (Kirby & Dacke, 1983; Jones et al , 1986; Shaw & Dacke, 1986; Gay, 1988). Although medullary bone is considered an evolutionary adaptation to help birds avoid a serious and potentially lethal negative calcium balance during reproduction (Miller, 1992),

there is considerable evidence that under conditions of limited calcium availability, the structural bone of the bird's skeleton is also resorbed (Urist, 1959; Simkiss, 1967; Zambonin-Zallone & Mueller, 1969). The results of this experiment, and of a previous experiment in this study, indicate that in the modern laying hen, medullary bone is maintained at the expense of cortical and cancellous bone during the normal course of egg production, without any evidence of calcium deficiency. The inability of bisphosphonate (of the type and at the dose administered) to prevent this structural bone loss may be a result of the relentless osteoclastic activity brought about by the egg-formation cycle. Also, reduction in structural bone mass during the laying cycle in alendronate-treated birds may be brought about by resorption of bone unbound by bisphosphonate. These may occur through the lack of alendronate binding to surfaces other than those prepared for resorption, or may be a reflection of the amount of structural bone formed after alendronate administration but before medullary bone modelling, which would obviously not be resistant to remodelling. It may be that the intensity of resorption during medullary bone remodelling is greater than during modelling, and that a greater proportion of unbound surfaces are subsequently resorbed.

Fluorochrome bone labels given to all mid-lay birds at the time of alendronate treatment persisted in some areas of cancellous bone, but were more fragmented. There was no apparent difference between the persistence of the labels in either group, but one possible explanation for this is that alendronate may not have bound to these formation surfaces. A third label, xylenol orange, administered 3 days prior to sacrifice was observed to be abundant in medullary bone spicules but absent from cancellous and cortical bone. This confirms an earlier finding of this study, that only medullary bone is formed during reproduction in female hens.

In the mid-lay alendronate-treated group, it was observed that medullary bone osteoid was present in greater quantities than in controls. This, together with the fact that medullary bone volume was significantly lower in this group than controls, suggests a problem with availability of mineral. It may be that if the number of potential sites of resorption in cancellous bone is reduced by the alendronate treatment, resorption of medullary bone will

be greater and will slow down the accretion of medullary bone through the laying cycle. Also, resorption and matrix formation occur simultaneosly in medullary bone (van de Velde et al,1984, 1985), while matrix mineralsation occurs later. A reduction in potential resorption sites may compromise the availability of mineral for this purpose.

In conclusion, the following points can be made;

1. Alendronate administration during lay is unlikely to prevent structural bone loss, and may have a deleterious effect on it because of its influence on calcium mobilisation from medullary bone.

2. Alendronate administration to pullets before ovarian follicle development prevented the structural bone loss associated with medullary bone modelling.

3. Alendronate administration to pullets did not prevent the structural bone loss associated with medullary bone remodelling.

The results from these experiments suggest that there is a potential role for bisphosphonates in preventing the osteoporosis typical of laying hens. More work is required to establish the optimum dose, identify the most effective bisphosphonates, and investigate binding sites in birds. However, it is clear that the timing of the bisphosphonate administration is crucial to its beneficial anti-resorptive effect in hens.

1.) Medullary bone is widespread throughout the skeleton of the mature female modern domestic fowl. In bones such as the femur it is present in large amounts, while in the pneumatised bones its development appears to be limited by the presence of the air sac. In pneumatised bones medullary bone is often formed within resorption cavities in existing cancellous and cortical bone, resulting in severe erosion of the latter towards the end of the egg-laying cycle. The toe was the only bone sampled which did not contain medullary bone.

2.) Medullary bone is present in all birds which have follicular development and appears to increase in volume during the laying cycle. When follicular development stops, medullary bone is resorbed but not completely; remnants of medullary bone remain on lamellar bone surfaces and become sandwiched between these and subsequent newly formed lamellae.

3.) Despite its woven nature, medullary bone in the modern laying fowl cannot be considered a temporary tissue, because it is present and intensively remodelled for most of the bird's life. The relationship between prolonged periods of intense medullary bone formation and cortical and cancellous bone loss required investigation.and quantification.

4.) In pullets,it was found that ovarian follicle development coincides with increases in plasma oestrogen and calcium, and results in cellular changes at the endosteal bone surface which ultimately lead to the formation of medullary bone at these sites. Histomorphometric measurements indicated that medullary bone is modelled during ovarian follicle development and subsequently remodelled in increasing volume during the egg-laying cycle.

4.) Medullary bone modelling resulted in significant decreases in cancellous bone volume. During medullary bone remodelling cancellous bone volume decreases further and cortical bone is eroded from the endosteal surfaces. There is no further mineralisation of cortical or cancellous bone after the onset of egg-production; fluorochrome bone labels bind only to medullary bone.

5.) There is a strong relationship between medullary bone formation and the loss of cancellous and cortical bone which subsequently results in an unnacceptable incidence of bone fracture in the modern laying hen. Genetic selection and improvements in husbandry have resulted in a laying cycle of greatly increased duration, and consequently increased the period of medullary bone remodelling. Therefore a system which has evolved to supply calcium for egg-shell formation without damaging the structural integrity of the skeleton has become the mechanism of its destruction.

6.) Medullary bone is formed under the influence of oestrogen and the role of the latter in the cancellous and cortical bone loss which occurs in hens was investigated. Oestrogen administered to male laying strain fowl resulted in medullary bone formation and significant cancellous bone loss. Conversely, an anti-oestrogen adminisered to female pullets prevented medullary bone formation and cancellous bone loss. Although it is unknown whether or not oestrogen directly or indirectly brings about cancellous bone loss in the fowl, it is clear that its role in the development of avian osteoporosis is entirely different to that in mammals. The presence of oestrogen initiates cancellous and cortical bone loss in birds, while in mammals the withdrawal of oestrogen is associated with bone loss.

7.) Osteoporosis in laying hens is associated with reproductive activity, and the most probable way of preventing it is to inhibit resorption of cancellous and cortical bone. The bisphosphonates are compounds which prevent bone resorption, and administration during ovarian follicle development resulted in significantly higher cancellous bone volume at point of lay than in controls. Although bisphosphonate treatment also resulted in significantly higher bone volumes mid-way through the laying cycle than in controls, both groups had lower cancellous bone volumes mid-lay than at the onset of lay and cortical bone loss was not prevented. Therefore bisphosphonates administered during follicular development prevented the cancellous bone loss associated with medullary bone modelling but did not prevent the cancellous and cortical bone loss associated with medullary bone remodelling. It is clear that bisphosphonates are effective in preventing bone resorption in birds and if administered at the onset of lay may be potentially useful in preventing osteoporosis in the modern commercial

FUTURE WORK

Results from the first experiment in this study indicated that medullary bone was more widespread and was present for longer in the skeleton of the modern laying hen than it was 30 years ago. The effects of medullary bone formation on bone strength require investigation; medullary bone, despite its woven nature, may have a previously unknown structural role. The interaction of the different bone types in determining strength would be an interesting area of study. Also, medullary bone formation in pneumatised bones differed from that in non-pneumatised bones and may subsequently compromise skeletal integrity to a greater extent. The question of whether pneumatised bones are rendered more fragile by medullary bone remodelling could be tested using machined samples of pneumatised humeri before and after medullary bone formation. A comparison of strength of pneumatised and non-pneumatised thoracic vertebrae (for example the fifth with the sixth) would also be useful. It was observed that medullary bone was not completely resorbed in out of lay hens and that remnants became sandwiched between existing cancellous bone surfaces and newly formed cancellous bone lamellae. The effects of this incomplete medullary bone resorption on the mechanical properties of the bone need further study.

The second experiment in this study concluded that ovarian follicular development coincided with increases in plasma calcium and oestrogen and resulted in cellular changes, including increased resorption, at the endosteal bone surfaces which ultimately led to the formation of medullary bone at these sites. Although much is known about the role of parathyroid hormone in bone resorption during the laying cycle, nothing is known about its role in relation to the bone changes which occur in the period before lay. The oestrogen-induced medullary bone formation model in male birds may be useful in investigating the relationships between oestrogen, parathyroid hormone, plasma calcium and the increased osteoclast numbers which bring about bone resorption in pullets. There may be other factors involved in recruiting and activating osteoclasts at this time and these also require study.

It was also clear from the second experiment in this study that medullary bone was present in a larger volume in point-of -lay pullets 30 years ago than in the modern pullet. This may have been influenced by intense selection which has brought about an earlier onset of lay. A study investigating differences in cancellous and medullary bone volumes at point of lay between

different strains of birds or between early- and late-laying individuals of the same strain would be of interest.

The third experiment indicated that oestrogen plays a different role in bone loss in birds than it does in mammals; the presence of oestrogen initiates cancellous and cortical bone loss in birds while in mammals the withdrawal of oestrogen is associated with bone loss. Avian medullary bone cells are widely used in in-vitro bone cell studies and have been previously considered similar to their mammalian counterparts. More comparative study of the effects of oestrogen on bone turnover in birds and mammals is essential before any such conclusions can be drawn.

Medullary bone is thought to be a uniquely avian phenomenon, and its function is believed to be the rapid provision of large quantities of calcium for egg-shell formation. Mammals can also be subjected to periods of high calcium demand (for example during antler formation in deer, or during prolonged periods of lactation), but none have adapted by laying down stores of woven bone. Reptiles also lay calcified eggs but do not form medullary bone. A study of the effects of calcium demand on bone turnover and the control of resorption in birds, mammals and reptiles would be fascinating.

The final experiment of this study showed that the bisphosphonate Alendronate administered during follicular development prevented the cancellous bone loss associated with medullary bone modelling but did not prevent the cancellous and cortical bone loss associated with medullary bone remodelling. Mammalian studies involving [^{3}H] Alendronate have determined that it binds preferentially to sites prepared for resorption and that its subsequent local release by osteoclastic acidification prevents ruffled border formation and resorption. This type of study is required to determine whether Alendronate's uptake and action on avian bone is similar, and would also be beneficial in determining more precisely the optimum dosing time. The experiment in this study used a low dose of alendronate; repeating the experiment at a higher dose may result in improved anti-resorptive effects during medullary bone remodelling.

Alendronate is one of many bisphosphonates which all vary in their potency to inhibit bone

resorption. In rats CGP 42'446 has more than 10 times the potency of alendronate. It is possible that such more powerful bisphosphonates may be better suited to resisting the relentless resorption experienced by avian bone during the egg-laying cycle. Their efficacy in preventing osteoporosis in laying hens merits further investigation.

BIBLIOGRAPHY

Adami S Salvagno G Guarerra G Montesanti F Garavelli S Rosini S Cascio VI (1986) Treatment of Paget's disease of bone with intravenous aminohydroxybutane bisphosphonate Calcified Tissue International 39 226-229

Aitken JM Hart DM Anderson JB Lindsay R & Smith DA (1973) Osteoporosis after oophorectomy for non-malignant disease British Medical Journal 1 325-8

Amprino R & Engstrom A (1952) Studies on X-ray absorpion and diffraction in bone tissue Acta Anat 15 1-22

Anderson FC Cope CD Crilly RG Hodsman AB & Wolf BM (1984) Preliminary obsevations of a form of coherence therapy for osteoporosis Calcified Tissue International 36 341-343

Anon (1983) Tamoxifen The Lancet 1199-1200

Antillon A Scott ML Krook L & Wasserman R (1977) Metabolic response of layhing hens to different dietary levels of calcium, phosphorus and vitaminD Cornell Veterinarian 67 413-444

Arnett R Lindsay R Dempster DW (1986) Effect of oestrogen and anti oestrogen on osteoclast activity in vitro Journal of Bone & Mineral Research 1 99

Ascenzi A Francois C & Bocciarelli DS (1963) On the bone induced by oestrogen in birds Journal of Ultrastructural Research. 8 491-505

Ascenzi A & Bell GH (1972)In: Biochemistry & Physiology of Bone, Vol. I(Ed GH Bourne)pp 311-352 Academic Press New York & London

Attardo-Paranello G Merlini G Pavesi F Cremaa F Fiorentini ML Ascari E (1987) Effects of a new amino diphosphonate in patients with osteolytic lesions from metastases and myeloatosis: comparison with dichloromethylene diphosphonate Archives International Medicine 147 1629-1633

Bacon WL Brown KI Musser MA (1980) Changes in plasma calcium phosphorus lipids and oestrogen in turkey hens with reproductive status Poultry Science 59 444-448

Banks WJ Epling GP Kainer RA Davio RW (1968) Antler growth and osteoporosis 1. morphological and morphometric changes in costal compacta during the antler growth cycle Anatomical Record 162 387-398

Baron R. (1977) Importance of the intermediate phases between resorption and formation in the measurement and understanding of the bone remodelling sequence. In: Bone Histomorphometry; proceedings of the 2nd international workshop (Ed, Meunier P) Toulouse: Societe de la Nouvelle Imprimerie Fournie pp 179-83

Bastien RW Bradley JW Pennington BL and Ferguson TM (1979) Effect of dietary mineral supplements on radius breaking strength and egg characteristics of cage layers. Poultry Science 58 90-92

Bellairs A & Jenkin CR (1960) In Biology and Comparative Physiology of Birds Volume 1 (Ed. A.J. Marshall), pp241-295. Academic Press

Belanger LF Choquette LPE Cousineau JG (1967) Technical approaches leading to the concept of osteocytic osteolysis Calcified Tissue Research 1 37

Belanger LF (1969) Osteocytic osteolysis Calcified Tissue Research 4 1

Belanger LF & Copp DH (1972) The skeletal effects of prolonged calcitonin administration in birds, under various conditions. In: Calcium, Parathyroid Hormone and the Callcitonins. Proceedings 4thParathyroid Conference. (Ed: R V Talmage and P L Munson), pp 41-50. Amsterdam: Excerpta Medica.

Bell GH (1959) Ann Med Perugia 50 355

Bell GH (1969) Advan Sci 26 75

Bell DJ & Sillar WG (1962) Cage layer fatigue in brown leghorns Research in Veterinary Science 3 219-230

Bickerstaff DR O'Doherty DP McCloskey EV Hamdy NAT Mian M Kanis JA (1991) Effects of aminobutylidene diphosphonate in hypercalcaemia due to malignancy Bone 12 17-20

Bloom MA & Domm LV (1941) Medullary bone in the pigeon Anatomical Record 81 91

Bloom W Bloom MA & McLean FC (1941) Medullary bone changes in the reproductive cycle of female pigeons Anatomical Record 81 443-475

Bloom MA McLean FC & Bloom W (1942) Calcification and ossification Anatomical Record 83 99-121.

Bloom MA Domm LV Nalbandov AV and Bloom W (1958) Medullary bone of laying chickens. American Journal of Anatomy 102 411-453

Boelkins JN & Kenny AD (1973). Plasma calcitonin levels in Japanese quail. Endocrinology 92 1754-1760.

Bowman BM & Miller SC (1986) The proliferation and differentiation of the bone lining cell in oestrogen-induced osteogenesis. Bone 7 351-357

Bouvier M & Hylander WL (1981) Effect of bone strain on cortical bone structure in macaques Journal of Morphology 167 1-12

Brown JP Delmas PD Arlot M Meunier PJ (1987) Active bone turnover of the cortico-endosteal envelope in post menopausal osteoporosis Journal of Clinical Endocrinology and Metabolism 64 954-959

Burssens A Gertz GJ Francis RM Tucci JR and Singer FR (1990) A double-blind, placebo controlled, rising multiple dose trial of oral alendronate in Paget's Disease Journal of Bone & Mineral Research. 5 S2

Candlish J K (1971) The collagen fibrils in fowl medullary bone. British Poultry Science 12 111-117.

Caputo CB Meadows D Raisz LG (1976)failure of oestrogens and androgens to inhibit bone resorption in tissue culture Endocrinology 98 1065- 1068

Carasco, MG de Vernejoul MC Sterkers Y Morieux CC Kuntz D & Miravet L (1989) Decreased bone formation in osteoporotic patients compared with age-matched controls. Calcified Tissue International 44 173-5

Castillo L Tanaka Y DeLuca HF Sunde ML (1977) The stimulation of 25 hydroxy-vitamin D3 1a hydroxylase by oestrogen Archives of Biochemistry and Biophysics 179 211-217

Chambers TJ & Fuller K (1985) Bone cells predispose bone surfacews to resorption by

exposure of mineral to osteoclastic contact Journal of Cell Science 76 155

Chappel BM (1978) On the relative density of avian and mammalian bones Journal of Anatomy 137 216-220

Chen TL & Feldman D (1978) Distinction between alpha feto protein and intracellular oestrogen receptors: evidence against the presence of oestradiol receptors in rat bones Endocrinology 102 235-239

Churches AE & Howlett CR (1981) The response of mature cortical bone to controlled time varying loading In:Mechanical Properties of bone (Ed SC Cowin) AMD New York American Society of Engineers pp69-80

Cohen JC & Harris WH (1958)The three dimensional anatomy of Haversian systems Journal of Bone & Joint Surgery 40 419-434

Common RH (1933) Observations on the mineral metabolism of pullets. Journal of Agricultural Science, 23, 555-570

Common RH Rutledge WA Hale RW (1948) The formation of medullary bone in hens Journal of Agricultural Science 38 64

Couch JR (1955) Cage layer fatigue Feed Age 5 55-57

Currey JC (1984) The mechanical adaptation of bones. Princeton, N.J. Princeton University Press

Dacke GC Musacchia XJ Volkert WA Kenny AD (1973) Comparative Biochemistry and Physiology 44A 1267-1275

Dacke CG (1979) In: Calcium regulation of sub-mammalian vertebrates. pp 156-162 Academic Press, London New York

Dacke CG Arkle S Cook DJ Wormstone I M Jones S Zaidi M and Bascal ZA (1993) Medulary bone and avian calcium regulation. Journal of Experimental Biology 184 63-88.

Dallemagne MJ (1948) The theory of primary calcification in bone Nature 161 115-117

Davidson NE (1992) Tamoxifen- panacea or Pandora's box new England Journal of Medicine 326 885-886

Delaisse JM Eeckhout Y Vaes G (1988) Bone resorbing agents affect the production and distribution of procollagenase as well as the activity of collagenase in bone tissue Endocrinology 123 264-276

Dequeker J Nijs J Verstraetem A Geusens P & Gevers G (1987) Genetic determinants of bone mineral content at the spine and radius; a twin study. Bone 8 207-9.

Doyle LP (1925) Rickets in mature chickens Poultry Science 4 146-150

Driggers JC & Comar CL (1949) The secretion of radioactive calcium in the hens egg. Poultry Science, 28, 420-424

Eastell R Delmaas PD Hodgson SF Eriksen EF Mann KG and Riggs BL(1988) Bone formation rate in older women; concurrent aassessment with bone histomorphometry, calcium kinetics, and biochemical markers. Journal of Clinical Endoocrinology & Metabolism 67 741-8.

Edgren RA (1960)A seasonal change in bone density in female musk turtles, sternothaerus

odoratus (latreille). Comparative Biochemistry and Physiology. 1 213-217.

Einhorn TA(1992)Bone strength: the bottom line Calcified Tissue International 51 333-339

Elsey RM & Wink CS (1985) Femoral bone as a possible source of calcium for eggshell deposition in Alligator Mississippiensis Anatomical Record 211 57A.

Elsey RM & Wink CS (1986) The effects of estradiol on plasma calcium and femoral bonre structure in alligators (alligator missipienssis) Comparative Biochemistry and Physiology 84A 107-110.

EnlowDH & Brown SO (1956) A comparative histological study of fossil and recent bone tissues. Texas Journal of. Science Part I 8 405-443

Enlow DH and Brown SO(1957). A comparative histological study of fossil and recent bone tissues. Texas .Journal of Science Part 2 9 186-214,

Enlow DH and Brown SO (1958). A comparative histological study of fossil and recent bone tissues. Texas .Journal of Science. Part 3 10, 187-230,

Eriksen EF Colvard DS & Berg NJ (1988) Evidence of estrogen receptors in normal human ossteoblast-like cells. Science 241, 84-86

Etches R.J (1987) Calcium logistics in the laying hen. Journal of Nutrition 117 619-628

Farner DS (1966) Photoperiodic control of the reproductive cycle in birds Scientific Progress 15 63-92

Fedducia A (1975) In: *Anatomy of the domestic animals* (Ed. Getty, R.), Vol. 2, pp 1786-1801. W.B. Saunders Co.

Feinblatt JD (1982) The comparative physiology of calcium regulation in sub-mammalian vertebrates. Advances in Physical Biochemistry 8, 73-110.

Feldman S Minne HW Parvizi S (1989) Antiestrogen and antianddrogen administration reduce bone mass in the rat Bone Mineral 7 245-254

Fleisch H (1993) Bisphosphonates in Bone Disease Stampfli Ltd Berne

Foote JS (1916) A contribution to the comparative histology of the femur Smithsonian Contributions to Knowledge 35 1-242

Francis DW (1957) Strain differences in the incidence of cage layer fatigue Poultry Science 36 181-183

Frost HM (1973) Bone remodelling and its relationship to bone metabolic disease chaarles C thomas, Sprinfield IL

Frost HM (1981) in: Osteoporosis (Eds. Deluca HF, Frost HM, Jee WSS, Johnson, CR & Parfitt, AM) pp 185-190 University Park Press, Baltimore

Frost HM (1986) Intermediary organisation of the skeleton, Vols 1 & 11 CRC Press

Furr BJA Paterson JS Richardson D Slater SR Wakeling AE (1978). In; Pharmacological and Biochemical Properties of Drug Substances Vol. 2 (Ed. ME Goldberg) pp 355-399. A.P.A. Washington.

Gardiner T (1964) Cage layers fatigue Agriculture Northern Ireland 39 81

Gay CV (1988) Avian bone resorption at the cullular level. C R C Critical Reviews in Poultry Biology. 1 197 - 210

Gibson WWC (1966) Cage Layer Fatigue Veterinary Record 78 910-911

Govaerts J & Dallemagne MJ (1948). Influence of folliculin on bone metabolism studied by means of radiophosphorus. Nature 161 977.

Gotfresden A Christiansen C Palshof T (1984) The effect of tamoxifen on bone mineral content in premenopausal womwn with breast cancer Cancer 53 853-857

Gregory NJ & Wilkins LJ (1989) Broken bones in domestic fowl: handling and processing damage in end of lay battery hens. British Poultry Science 30 355-362

Gregory N.J & Wilkins LJ (1992) In: Bone Biology and Skeletal Disorders of Poultry (Ed CC Whitehead) pp 313-328. Carfax Publishing Co.

Grumbles LC (1959) Cage layer fatigue (cage paralysis) Avian Diseases 3 122- 125

Guy JA Shea M Peter CP Morrissey R Hayes WC (1993) Continuous alendronate treatment throughout growth, maturation and ageing in the rat results in increases in bone mass and mechanical properties Calcified Tissue International 53 283-288

Guyer RB Grunder AA Buss EG Clagett CO (1980) Calcium binding proteins in theserum of chickens: Vitellogenin and albumin Poultry Science 59 874-877

Harland HC (1927) Genetic differences in egg-laying fowl Journal of Genetics 18 55-62

Harms RH (1962) Feed Age 12 26-29

Hartwigk H (1966) Kafigmudigkeit oder Kafiglahme des Legehuhnes ein neues Syndrom bei im Kafig gehaltenen Huhnern Deutsch Tieraerztl Wochenschr 73 277- 279

Heaney RP & Saville PD (1976) Etidronate disodium in post menopausal osteoporosis. Clinical Pharmacol Therap 20 593-604

Hert J Pribylova E Liskova M (1972) Microstructure of compact bone after intermittent loading Acta Anat 82 218-230

Hildebrand M (1972) Analysis of vertebrate structure J Wiley and son , New York

Hodges RD (1974) The histology of the fowl Academic Press London, New york

Hodsman AB (1989) Effects of cyclical therapy for osteoporosis using an oral regimen of inorganic phosphate and sodium etidronate; a clinical and bone histomorphometric study. Bone Mineral 5 201-12

Hogg DA (1984a) The distribution of pneumatisation in the skeleton of the adult domestic fowl Journal of Anatomy 138 617-629

Hogg DA (1984b) The development of pneumatisation in the post cranial skeleton of the domestic fowl Journal of Anatomy 139 105-113

Holtrop ME (1990) In; Bone (vol. 1) (Ed. B K Hall). pp 1 -39 Telford Press

Holtrop ME (1990) In; Bone (vol. 2) (Ed. B K Hall). pp 1 -29 Telford Press

Hughes BO and Appleby MC (1989) Increase in bone strength of spent laying hens housed in modified cages with perches.Veterinary Record 124 483-484

Hughes DE Mian M Guillland-Cumming DF Russell RGG (1991) The cellular mechanism of action of bisphosphonates. Drugs Under Experimental Clinical Research XVII, 2 109-114.

Hughes BO Wilson S Appleby MC & Smith SF (1993) Comparison of bone volume and strength as measures of skeletal integrity in caged laying hens with access to perches. Research in Veterinary Science 54 202-206

Hurwitz S (1964) Calcium metabolism of pullets on the onset of egg production as influenced by dietary calcium level. Poultry Science 43 1462 - 147

Hurwitz S (1965) Calcium turnover in different bone segments of laying fowl. American Journal of Physiology 208 203-207

Hurwitz (1992) In: Bone biology and skeletal disorders of poultry (Ed. CCWhitehead.) pp 87-102. Carfax Publishing Co.

Huxley, T.H. (1871) *Anatomy of vertebrated animals*. Churchill.

Innoue M (1966) Histological studies of the medullary bone in the femur of domestic fowl Japanese Journal of Veterinary Science 28 161-181

Jacoby S Snapir N Rozenboim I Arnon E Meidan R & Robinzon B (1992) Tamoxifen advances puberty in the white leghorn hen British Poultry Science 33 101-111

Jande SS and Belanger LF (1973). The life cycle of the osteocyte. Clinical Orthopaedics and Related Research 94 281-305.

Jee WSS (1988) In: Cell and Tissue Biology (Ed Weiss L) pp213-253 Urban & Schwaszenberg Baltimore

Johnson AL & van Teinhoven A (1980) Plasma concentrations of six steroids and LH during the ovulatory cycle of the hen Biology & Reproduction 23 910-916

Johnson, A.L. (1986) In: *Avian Physiology* (Ed. PDSturkie), pp 403-431. Berlin, Springer-Verlag.

Johnson CC Melton LJ Lindsay R Eddy DM (1989) Clinical indications for bone mass measurements Journal of Bone and Mineral Research 4 (Suppl)2

Johnston CC Hui SL Witt RM Appledorn R Baker RS and Longscope C (1984) Early menopausal changes in bone mass and sex steroids. Journal of Clinical. Endocrinology & Metabolism 61 905-11.

Jones SJ & Boyd A, (1976) Experimental studies on changes in osteoblastic shaape induced by calcitonin and parathyroid extract in an organ culture system Cell Tissue Research 169 449-465

Jones SJ Ali NN Boyde A (1986) Survival and resorptive activity of chick osteoclasts in culture Anat Embryol 174 265-272

Kanders B Dempster DW and Lindsay R(1988) Interaction of calcium, nutrition and physical activity on bone mass in young women. Bone & Mineral Research 3, 145-149.

King, AS & King DZ (1979) In: Form and function in birds (Eds. King, A.S. & M^{c}Clelland, J.) pp 23-35. London, Academic Press.

King AS (1957) The aerated bones of gallus domesticus Acta Anat 31 220-230

Kirby GC & Dacke CG (1983) Hypercalcaemic responses to 16,16-dimethyl prostaglandin E2, a stable prostaglandin E2 analogue in chicks. Journal of Endocrinology 99 115-122.

Kleerkoper M Villaneuva AR Stanciu Rao DS and Parfitt AM. (1985) The role of three dimensional trabecular microstructure in the pathogenesis of vertebral compression fractures. Calcified Tissue International 37, 594-597.

Knowles TG & Broom DM (1990) Limb bone strength and movement in laying hens from different housing systems. Veterinary Record 126 354-356.

Kusuhara S and Schraer H.(1982). Cytology and autoradiography of estrogen-induced differentiation of avian endosteal cells. Calcified Tissue International. 34 352-358.

Kyes P & Potter TS (1934). Physiological marrow ossification in female pigeons. Anatomical Record 60 377-379,

Lacroix P (1971)The internal remodelling of bones In: The Biochemistry and Physiology of Bone Vol 3 (Ed GH Bourne) pp 119-138 Academic Press, London. New York

Landauer W Pfeiffer CA, Gardner WU Shaw JC (1941) Blood serum and skeletal changes in two breeds of ducks receiving estrogens.

Landauer W and Zondek B (1944). Observations on the structure of bone in estrogen-treated cocks and drakes. American Journal of Pathology. 20 179-208.

Lanyon (1992) In: Bone biology and skeletal disorders of poultry (Ed. CCWhitehead) pp 61-66. Carfax Publishing Co.

Laven H (1940) Uber nachlegen und weiterlegen Ornithologie Monatsber 48 131-136

Legha SW (1988) Tamoxifen in the treatment of breast cancer Ann Intern Med 109 219-228

Lindsay R & Cosman F (1992) In: Disorders of Bone and Mineral Metabolism (Eds. FL Coe & MJ Favus) pp 831-888 Raven Press New York

Lofts B & Murton RK (1973) In: Avian Biology (Eds. Farner DS & King, JR) Vol. 3 pp 1-107 London, Academic Press.

Love R Mazess RB Barden S Epstein S Newcombe PA Jordan VC Carbone PP and DeMets DL (1992) Effects of tamoxifen on bone mineral density in postmenopausal women with breast cancer. The New England Journal of Medicine 326 852-856.

Loveridge N Thomson BM & Farquharson C (1992) In: Bone biology and skeletal disorders of poultry (Ed. CC Whitehead) pp 3-18 . Carfax Publishing Co.

Maller M, Horsman A Harvald B Hauge .M Henningsen K and Nordin BEC.(1978) Metarpal morphometry in monozygotic and dizygotic elderly twins. Calcified Tissue Research. 25 197-201.

MacOwan MM (1932) Hypertrophy of the parathyroid glands at the onset of reproduction in laying hens Quarterly Journal of Experimental Physiology 21 383-392

Marcus R (1987) Normal and abnormal bone remodelling in man Annual Reviews in Medicine 38 129-141

Marie PJ Sabbagh A de Vernejoul MC and Lomri A.(1989) Osteocalcin and deoxyribonucleic acid synthesis in vitro and histomorphometric indic es of bone formation in postmenopaaausal osteoporosis. Journal of Clinical Endocrinology & Metabolism 69, 272-9.

Marotti G (1976). Decrement in volume of osteoblasts during osteon formation and its effect on the size of the corresponding osteocytes. In: Bone Histomorphometry. (Ed. P J Meunier) pp 385-397.

Martin TJ Raisz LG and Rodan G. In: Clinical endocrinology of calcium metabolism.(Eds. Martin TJ Raisz LG) New York; Marcel Dekker (1987), 1-52

Matkovic V Tominac C Fontana D and C hestnut C.(1988) Influence of calcium on peak bone mass: a 24-month follow-up. Journal of Bone & Mineral Research 3 (suppl. 1) S85,

McGregor RR Chu LLH Hamilton JW and Cohn DV (1973). Partial purification of parathyroid hormone from chicken parathyroid glands. Endocrinology 92 1312-1318

McClelland J (1989) In: Form and Function in Birds Volume 4 (Eds J McClelland & AS King) pp250-279 Academic Press London & New York

McSheeyhy PMJ & Chambers TJ (1986) Osteoblast-like cells in the presence of parathyroid hormone release soluble factor that stimulates osteoclastic bone resorption Endocrinology 119 1654-1659

Meister W (1951) Changes in the histological structure of the long bones of birds during the molt. The Anatomical Record, 111 1-21.

Meunier P Aaron J Eduoard C Vignon G (1971) osteoporosis and the replacement of cell populations of the marrow by adipose tissue clinical Orthopaedics and Related Research 80 147-154

Miller SC (1977) Osteoclast cell surface changes during egg laying cycle in Japanese quail. Journal of Cell Biology, 75 104-118

Miller SC (1981) Osteoclast cell surface specializations and nuclear kinetics during egg-laying in Japanese quail, American Journal of Anatomy, 162, 223-231

Miller SC & Bowman BM (1981) Medullary bone osteogenesis following estrogen administration to mature male Japanese quail. Developmental Biology 87 52-63.

Miller SC (1985) The rapid appearance of acid phosphatase activity at the developing ruffled border of parathyroid hormone activated medullary bone osteoclasts. Calcified Tissue International 37 526-529.

Miller S de Saint-Georges L Bowman BM and Jee WSS (1989). Bone lining cells; structure and function, Scanning Electron Microscopy 3 953-961.

Miller SC (1992) In: Bone biology and skeletal disorders of poultry (Ed. CC Whitehead) pp 103-116. Carfax Publishing Co.

Murray PDF (1936)Bones University Press Cambridge

Nayfield SG Karp JE Ford LG Dorr IA Kramer BS (1991) Potentia role of tamoxifen in prevention of breast cancer Journal of the National Cancer Institute 83 1450-1459

Nishida Y (1980)In: Biological Rhythms in Birds (Eds Y Tanable K Tandka K Ookawa) pp301-313 Japanese Scientific Society Press Tokyo

Norgaard-Neilsen G (1990) Bone strength of laying hens kept in an alternative system compared with hens in cages and on deep litter. British Poultry Science 31 81-89.

Nutek G & Cruess RL (1974) Estrogen receptors in bone: an evaluation of the uptake of estrogen into bone cells. Proceedings of the Society of Experimental Biology and Medicine. 146 265-268.

O'Doherty DP Bickerstaff EV McCloskey EV Hamdy NAT Beneton MNC Harris S Mian M and Kanis JA (1990). Treatment of Paget's disease of bone with amino-hydroxbutylidene bisphosphonate. Journal of Bone & Mineral Research 5 483-491.

Oshashi T Kusuhara S & Ishida K (1987) Effects of oestrogen and anti-oestrogen on the cells of the endosteal surface of male japanese quail. British Poultry Science 28 727-732

Oshashi T Kusuhara S & Ishida K (1988) Alkaline phosphatase activity during the process of cell differentiation on the endosteal surface of male Japanese Quail administered with oestrogen and anti-oestrogen Japanese Journal of Zootechnological Science 59 458-461

Oshashi T Kusuhara S & Ishida K (1990) Histochemical identification of oestrogen target cells in the medullary bone of laying hens. British Poultry Science 31 221-224

Ohashi T Kusuhara S and Ishida K (1991). Estrogen Target Cells during the early stage of meduallay bone osteogenesis' immunohistochemical detection of estrogen receptors in osteogenic cells of estrogen-treated Japanese quail. Calcified Tissue International 49 124-127.

Pacifici R Mc Murtry C Vered I Rupich R Avioli LV (1988) Coherence therapy does not prevent axial bone loss in osteoporotic women: a preliminary comparative study Journal of Clinical Endocrinology and Metabolism 66 7647-753

Paice C (1993) Edging towards a new welfare state Farmers Weekly January 42-44

Papapoulos SE Landman JO Bijvoet OLM Lowik CWGM Valkema R Pauwels EKJ & Vermeij P (1992) The use of bisphosphonates in the treatment of osteoporosis. Bone 13 S41-49

Papapoulos SE (1994) Bisphosphonates: introduction and rationale for their use in the treatment of osteoporosis Daavos Switzerland April 15 1994

Parfitt AM (1981) in: Osteoporosis (Eds. Deluca HF, Frost HM, Jee WSS, Johnson, CR & Parfitt, AM,) pp 115-126 University Park Press, Baltimore

Parfitt AM Mathews CHE Villanueva AR Kleerekopper M Frame B Rao DS (1983) Relationships between surface, volume and thickness of iliac trabecular bone in ageing and in osteporosis; implications for the microanatomic and cellular mechanisms of bone. Journal of Clinical Investigation 72 1396-1409

Parfitt AM & Kleerekoper, M (1984) Diagnostic value of bone histomorphometry and comparison of histologic measurements and biochemical indices of bone remodelling In: Osteoporosis (Eds Christiansen C, Arnaud CD, Nordin BEC Parfitt AM Peck WA Riggs BL)

Parfitt AM (1988) Bone remodelling in the pathogenesis of osteoporosis. Medical Times 109 80-92

Pedrazzoni M Palumerri E Pioli G (1989) Involutional osteoporosis and ADFR treatment; a controlled pilot study Current Therapeutic Research 45 188-197

Perrins AJ (1992) In: Bone biology and skeletal disorders of poultry (Ed. CC Whitehead) pp 345-348. Carfax Publishing Co.

Pfeiffer CA and Gardiner WU (1938). Skeletal changes and blood serum calcium level in pigeons receiving estrogens 23 485-491.

Pfeiffer CA and Kirschbaum A (1941) Secretion of androgen by the sparrow ovary following stimulation with pregnant mare serum Yale Journal of Biological Medicine 13 315-322

Phillips JG Butler PJ & Sharp PJ (1985) Physiological Strategies in Avian Biology pp Blackie

Pines M Bar A Hurwitz S (1984) Isolation and purification of avian parathroid hormone using high performance liquid chromatography, and some of its properties. General and Comparative Endoocrinology. 53 224-231.

Pocock NA Eisman JA Hopper JL Yeates MG Sambrook PN and Ebers S (1987). Genetic determinants of bone mass in adults. Journal of Clinical Investigation 80 706-10.

Pritchard JJ (1972)In: The Biochemistry and Physiology of Bone Vol I (Ed GH Bourne) pp 1-20 Academic Press London New York

Randall CJ & Duff SRI (1988) Avulsion of the patellar ligament in osteopenic laying fowl Veterinary Record 123 439-443

Raisz LG (1992) In: Disorders of Bone & Mineral Metabolism (Eds MJ Favus & FL Coe) pp287-312 Raven Press New York

Rasmussen H (1961) Parathyroid hormone: nature and mechanism of action American Journal of Medicine 30 112-128

Recker RR Kimmel DB Parfitt AM Davies KM Keshawarz NM Hinders SM (1988) Static and tetracycline-based bone histomorphometric data from 34 normal post-menopausal females Journal of Bone & Mineral Research 3 133-144

Recker RR (1992) In: Disorders of bone and mineral metabolism (Eds. FL Coe & MJ Favus) pp 219-240

Reginster JY Lecart MP Deroisy R Sarlet DD Ethgen D Collette J and Franchimont P (1989) The Lancet 1469-1471

Reilly DT & Burstein AH (1975) The elastic and ultimate properties of compact bone tissue Journal of Biomechanics, 8 893-405

Richelson LS Wahner HW Melton LJ and Riggs BL (1984). Relative contribtuions of aging and estrogen deficiency to postmenopausal bone loss. New England Journal of Medicine 311 1273-5.

Riddell C Helmboldt CF Singsen EP Matterson LD (1968) Bone pathology of birds affected with cage layer fatigue Avian Diseases 12 285-296

Riddell C Helmboldt CF Singsen EP (1969) A histological study of medullary bone of laying hens under different diet and housing conditions Avian Diseases 13 163-170

Riddell C. (1981) Skeletal disorders in poultry. Advances in Veterinary Science and Comparative Medicine 44 275-279

Riddell C (1992) In: Bone biology and skeletal disorders of poultry (Ed. CC Whitehead) pp 119-145. Carfax Publishing Co.

Riggs BL Wahner HW Seeman E (1982) Changes in bone mineral density of the proximal femur and spine with ageing. Journal of Clinical Investigation 70 716-723

Roland DA & Rao SK (1992) In: Bone biology and skeletal disorders of poultry (Ed. CC Whitehead) pp 281-295. Carfax Publishing Co.

Romanoff AL.& Romanoff AJ (1949) In: The Avian Egg, New York, Wiley Inc.

Rozenboim I Dgany O Robinzon B Arnon E and Snapir N (1989) The effect of tamoxifen on the reproductive traits in White Leghorn cockerels Pharmacology Biochemistry and Behaviour. 32 377-381.

Rubin CT and Lanyon LE (1988) Regulation of bone mass by mechanical strain magnitude. Calcified Tissue International 37 411-417.

Ruth ES (1918) Phillipines Journal of Science 13 311-318

Ryan WG, Wolter PH Bagdade J D (1991) Apparent beneficial effects of tamoxifen on bone mineral content in patients with breast cancer: preliminary study. Osteoporisis International 2 39-41

Salt GW & Zeuthen E (1960) In: Biology and Comparative Phsiology of Birds (Ed AJ Marshall) pp363-304 Academic Press London & New York

Sato M and Grasser WW (1990). Effects of bisphosphonates on isolated rat osteoclasts as examined by reflected microscopy. Journal of Bone & Mineral Research 5 31-40,

Sato M Grasser W Endo N Atkins R Simmons H ThompsonD Golub E & Rodan GA (1991) Alendronate localisation in rat bone and effects on osteoclast structure Journal of Clinical Investigation, 88, 2095-2105

Scheifer B & Dorn P (1969) Berl Muench Tieraerztl Wochenschr 82 151-153

Schenk R Eggli P Fleisch H and Rosini S (1986) Quantitative morphometric evaluation of the inhibitory activity of new aminobisphosphonates on bone resorption in the rat. Calcified Tissue International 38 342-349.

Schraer H and Hunter SJ (1985) The development of medullary bone; a model for osteogenesis 82 13-17.

Seedor J Quartuccio H Thompson D (1991) The bisphosphonate alendronate (MK-217) inhibits bone loss due to ovariectomy in rats Journal of Bone & Mineral Research 6 339-346

Sietsema WK Ebetino FH Saalvagno AM Bevan JA (1989) Anti-resorptive dose response relationships across three generations ofbisphossphonates Drugs Experimental Clinical Research 15 389-396

Shaw AJ & Dacke CG (1986) A comparison of the effects of bovine parathyroid hormone 1-43 and methylated prostaglandin E2 analogues on plasma calcium and inorganic phosphate levels in immature chickens and rats. General Comparative Endocrinology 61 164-171

Simkiss K & Tyler C (1959) The possible calcification mechanisms in some reptilian egg shells Quarterly journal of Microscopical Science 100 529-538

Simkiss K (1961) Calcium metabolism and avian reproduction. Biological Reviews, 36, 321-367

Simkiss K (1967) Calcium in Reproductive Physiology London, Chapman and Hall

Simmons DJ & Pankovitch AN (1964) Estrogen induced intra medullary bone formation in Japanese quail Endocrinology 74 646-648.

Simpson CF Waldroup PW Ammerman CB Harms RH (1964) Relationships of dietary calcium and phosphorus levels to the cage layer fatigue syndrome Avian Diseases 8 92-100

Smetana P (1965) Cage layer osteoporosis Journal of the Department of Agriculture of

Toft D & Gorski J (1966) A receptor molecule for estrogens: isolation from the rat uterus and preliminary characterisation Proceedings of the National Academy of Science U S A 55 1574-1581.

Tonna EA (1973) An electron microscopic study of skeletal cell ageing-II: the osteocyte. Experimental Gerontology 8 9-16.

Turken S Selldin D and Lindsay R. Effects of tamoxifen on spinal bone density. Journal of the National Cancer Institute (1989) 81 1086-8.

Turner RT & Schraer H (1977) Estrogen-induced sequential changes in avian bone metabolism. Calcified Tissue Research 24 157-162.

Turner RT Wakley GK Hannon KS Bell NH (1987) Tamoxifen prevents the skeletal effects of ovarian hormone deficiency in rats Journal of Bone and Mineral Research 2 449-456.

Turner RT Wakley GK Hannon KS Bell NH (1988) Tamoxifen inhibits osteoclast-mediated resorption of trabecular bone in ovarian hormone-deficient rats. Endocrinology 122 1146-1150

Turner RT Bell NH Gay CV (1993) Evidence that estrogen binding sites are present in bone cells and mediate medullary bone formation in Japanese quail. Poultry Science 72 728-740.

Urist M R (1959) The effects of calcium deprivation upon the blood, adrenal cortex, ovary, and skeleton in domestic fowl. In: Recent Progress in Hormone Research (Ed. G Pincus). pp 455-481. Academic Press New York and London..

Van de Velde JP Loveridge N & Vermeiden JPW (1984) Parathyroid responses to calcium avian medullary bone formation. Journal of Bone and Mineral Research 6 1249-1256

Van de Velde JP. Vermeiden JPW & Bloot AM (1985) Medullary bone matrix formation, mineralisation and remodelling related to the daily egg-laying cycle of the Japanese quail: a histological and radiological study. Bone 6 321-327

Veis A & Sabsay B (1987) In: Bone & Mineral Research Vol 5 (WA Peck, Ed) pp 1-64 Elsevier

Von Eggeling H (1938) In: Handbuch der vergleichenden Anatomie der Wirbeltiere (L. Bolk Ed.) pp 275-283 Urban & Schwarzenberg Berlin

Wakeley GK Hannon KS Bell NH Turner RT (1987) tamoxifen prevents the skeletal effects of ovarian hormone deficiency in rats Clinical Research 35 159A

Watts NB Harris ST Genaant HK Wasnich RD Miller PD Jackson RD Licataa AA (1990) Intermittent cyclical etidronate treatment of post-menopausal osteoporosis New England Journal of Medicine 323 73-79

Weidenreich F (1930) In; Haandbuch der mikroskopischen anatomie ded menchen (W von mollendorf Ed) pp 408-421 Springer Verlag Berlin,

Welty JC (1962) The Life of Birds Saunders Philadelphia

Whitehead, CC & Wilson S (1992) In: Bone biology and skeletal disorders of poultry (Whitehead CC, Ed.) pp 265-295. Carfax Publishing Co

Wideman RF (1990) Integrated calcium homeostasis in laying hens: an overview Proceedings of the AAAP Symposium San Antonio Texas.

Williams DC Paul DC and Herring, DD (1991) Effects of antiestrogenic compounds on avian medullary bone formation Journal of Bone & Mineral Research 6 1249-1256

Wilson JH & Harner JP (1988) Influence of body weight and cage height on the ultimate bending force and stress of the radius and tibia of layers Transactions of the American Society of Engineers 31 578-581

Wilson JH Mason JP & Beane WL (1990) Influence of calcium and phosphorus on bone strength of hens. Transactions of the American Society of Agricultural Engineers 33 642-647.

Wilson S & Duff SRI (1990) Morphology of medullary bone duriing the egg formation cycle.Research in Veterinary Science. 48 216-220.

Wilson S & Duff SRI (1991) The effects of vitamin or mineral deficiency on the morphology of medullary bone in laying hens. Research in Veterinary Science, 50, 216-221.

Wilson S Duff, SRI & Whitehead CC (1991) Effects of age, sex, and housing on the trabecular bone of laying strain domestic fowl. Research in Veterinary Science 53 52-58.

Wilson S Hughes BO Appleby MC & Smith SF (1993) Effects of perches on trabecular bone volume in laying hens. Research in Veterinary Science 54 207-211

Wood-Gush DGM (1971) The behaviour of domestic fowl. Heinemann

Yeager V L Chiemchanya S and Chaiseri P (1975) Changes in size of lucunae during the life of osteocytes in osteons of compact bone. Journal of Gerontology. 30 9-14.

Zambonin-Zallone A & Mueller W J (1969) Medulary bone of laying hens during calcium depletion and repletion. Calcified Tissue Research 4 136-146.

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The effect of a bisphosphonate on bone volume and eggshell structure in the hen

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SUMMARY

Bisphosphonates, used in the prevention and treatment of osteoporosis in man, can prevent bone loss in experimental models of osteoporosis in mammals. In egg-laying hens there is a high incidence of bone fractures which are due to osteoporosis. Alendronate, a bisphosphonate, was given to three groups of hens in mid-lay. Different doses of alendronate were given to each group and group 4 was a control. The birds were killed after 2 weeks of treatment. The hens receiving the highest dosage of alendronate (1 mg/kg every 2nd day) ceased laying and had reduced serum calcium concentrations. Lower dosages of alendronate (0.1 and 0.01 mg/kg every 2nd day) resulted in normal egg production and serum calcium concentrations. Egg shells with ultra-structural features indicative of reduced shell quality were produced by hens on the two higher dosages, but the egg shells from the controls and from the hens on the lowest dosage were considered normal. When alendronate was administered to hens in mid-lay there was no effect on trabecular bone volumes, but there was a reduction in mean medullary bone volume in some groups.

In a second experiment, pullets were treated with alendronate (0.01 mg/kg twice a week) before the onset of lay. The pullets were killed after laying their first egg. In the pullets treated with alendronate, this protocol resulted in a significantly greater volume of trabecular (structural) bone at the onset of lay.

INTRODUCTION

Hens producing a large number of eggs are prone to bone fractures that are attributed to osteoporosis (Riddell *et al.*, 1968). The incidence and severity of osteoporosis are unacceptably high, and approximately 30% of birds have fractures at the end of lay (Gregory & Wilkins, 1989).

In the egg-laying hen the marrow cavities of many bones contain medullary bone that serves as a source of labile calcium (Miller, 1977). Medullary bone is a form of woven bone, its formation is stimulated by the actions of oestrogens and androgens concomitant with the maturation of the ovarian follicles (Simkiss,

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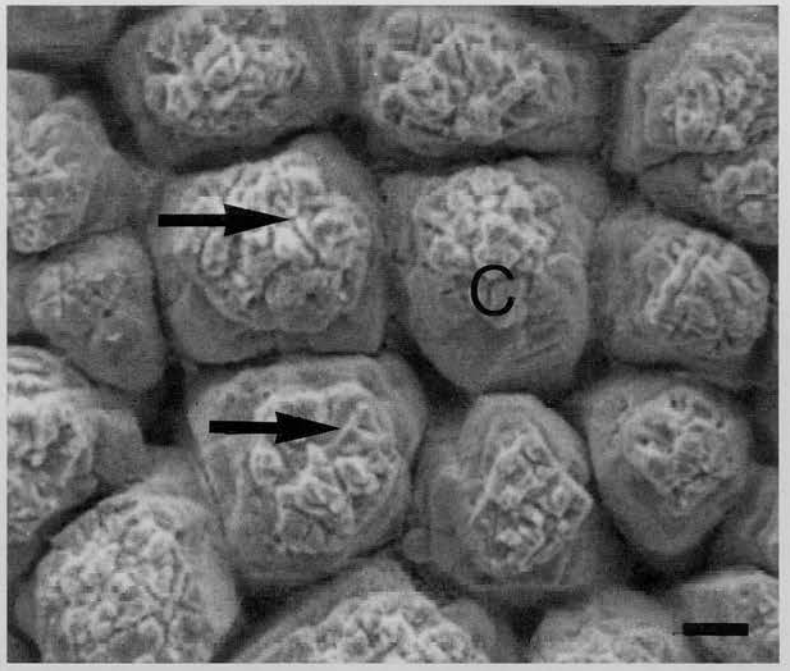

Figure 1. *The mammillary layer of an egg from a hen that was not treated with alendronate (experiment 1, group 4). There is variation in the size of individual mammillary bodies. The cap regions (c) are deeply etched (arrows) with the tracks formerly occupied by the membrane fibres. (Bar = 31 μm.)*

1967). Resorption of medullary bone provides 40% of the calcium required for shell formation (Meuller *et al.*, 1964). It has no proven structural role, unlike cortical and trabecular bone which maintains the structural integrity of the skeleton. The loss of these structural bone components leads to osteoporosis in laying hens and measurements of trabecular bone volume are used to assess the degree of bone loss (Wilson *et al.*, 1992). Rapid sequential changes from medullary bone resorption to formation occur with each egg laying cycle (Bloom *et al.*, 1958). Ultra-structural and light microscopy studies show that osteoid seams indicating bone formation and resorption lacunae indicating bone resorption, are present on medullary bone, but are very rarely found on trabecular bone (S. Wilson, manuscript in preparation) indicating minimal trabecular bone turnover. However, despite a minimal osteoclast presence on trabecular bone, histomorphometric studies show a decline in trabecular bone volume during lay (Wilson *et al.*, 1992), indicative of continuing resorption which increases the incidence and severity of osteoporosis. Recent studies indicate that in pullets, between the onset of ovarian follicular activity and the onset of lay, there is a marked reduction in trabecular bone volume (S. Wilson, manuscript in preparation).

Bisphosphonates, which are pyrophosphates characterized by a P-C-P bond,

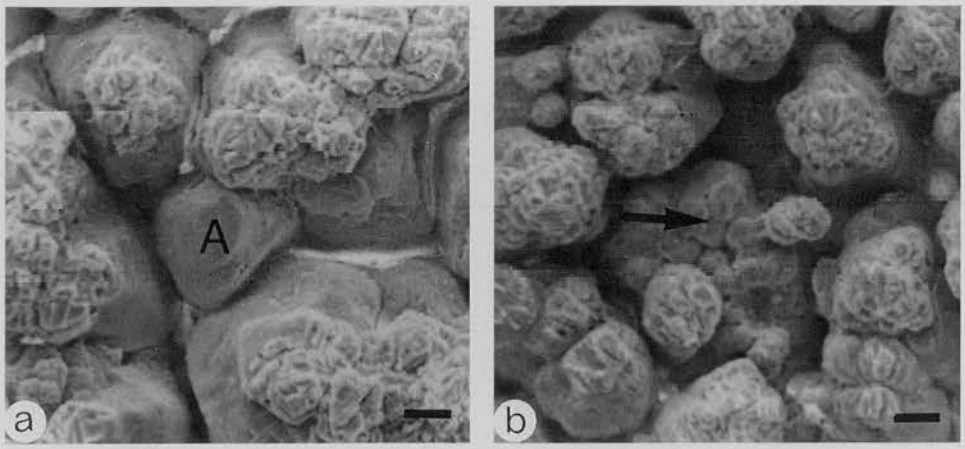

Figure 2. *The egg shells from birds receiving the highest dosage of Alendronate (experiment 1, group 1) displayed a variety of structural variations in the mammillary layer consistent with a reduction in shell quality. (a) Type A bodies which although making some contribution to the growth of the palisade layer, have minimum contact with the membrane fibres. There is a reduction in the effective thickness of the shell due to late fusion of the palisade columns. (b) Poorly etched caps (arrow) were also found and are indicative of minimal membrane contact. (Bar = 31 μm.)*

have a high affinity for hydroxyapatite and inhibit the resorption of bone (Fleish, 1987). These compounds are used clinically and appear to be effective in the prevention of bone loss in man (Elomma *et al.*, 1983; Minaire *et al.*, 1981). Their activity changes with the length and type of side-chains associated with the carbon atom (Shinoda *et al.*, 1983). Recent studies have shown that aminohydroxybutane bisphosphonate (alendronate) inhibits trabecular bone loss in immobilized (Thompson *et al.*, 1990) and ovariectomized (Seedor *et al.*, 1991) rats. This and other (Sato & Grasser, 1990) experimental work suggests that bisphosphonates function by impairing osteoclast function and that there is no impairment of osteoclast recruitment. However, nothing is known of the pharmacological efficacy of bisphosphonates in birds.

Trabecular bone has a greater surface area per unit volume than cortical bone and changes in trabecular bone volume are earlier and more marked. Trabecular bone volume measurements are, therefore, the preferred method for the assessment of osteoporosis in man (Marcus, 1991) and avians (Wilson *et al.*, 1992). The purpose of the present study was to establish if alendronate has pharmacological activity in egg-laying hens, and if it has a potential role in reducing the incidence and severity of osteoporosis.

MATERIALS AND METHODS

In the first experiment 20 mid-lay "Hisex" hens were housed in individual laying cages and eggs were collected daily. The birds were 39 weeks of age and had been in lay for approximately 17 weeks. They were fed a standard layer diet and water

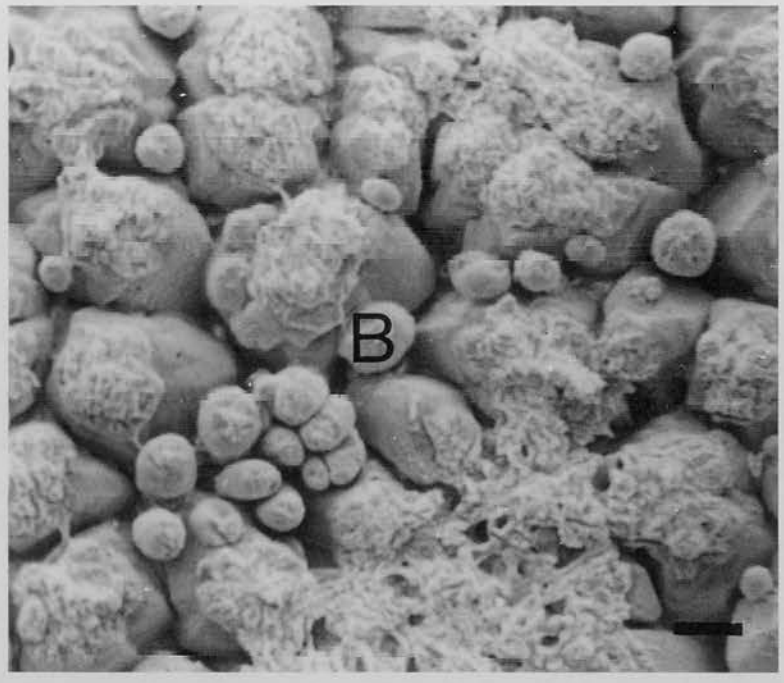

Figure 3. *The shells from the birds of experiment 1, group 2 have a more compact mammillary layer, which contains a high proportion of rounded type B bodies. These structures are flawed on two accounts: their lack of membrane contact and their failure to contribute to palisade layer formation. (Bar = 31 μm.)*

ad libitum. The hens were randomly divided into four groups of five individuals. Every second day for 2 weeks, three of the groups of hens received 1 ml/kg of sterile water containing alendronate by subcutaneous injections. The injections for group 1, 2 and 3, respectively, contained 1.0, 0.1 and 0.01 mg/ml. The birds in group 4 were only injected with sterile water and served as a control. Injections were made subcutaneously over the superficial pectoral muscle.

Egg laying was monitored and shells examined. Samples of egg shells from each bird were collected for examination by scanning electron microscope. Egg contents were removed by blowing, the interior was then washed with distilled water and pieces of shell excised from the equatorial region. The outer shell membrane was manually stripped and the adherent inner membrane removed by plasma etching (Reid, 1983). All samples were attached with silver paint to aluminium stubs, mammillary layer uppermost, coated with gold/paladium and viewed with a Philips 501B scanning electron microscope at 15KV.

Before starting treatment, during treatment and prior to killing, blood samples were collected and serum was prepared. Serum calcium, phosphorous and alkaline phosphatase levels were measured using clinical chemistry reagent packs (Wako, Alpha Laboratories Ltd, Hampshire). In avian serum, alkaline phosphatase values are much higher than those found in man (M. Mitchell, personal

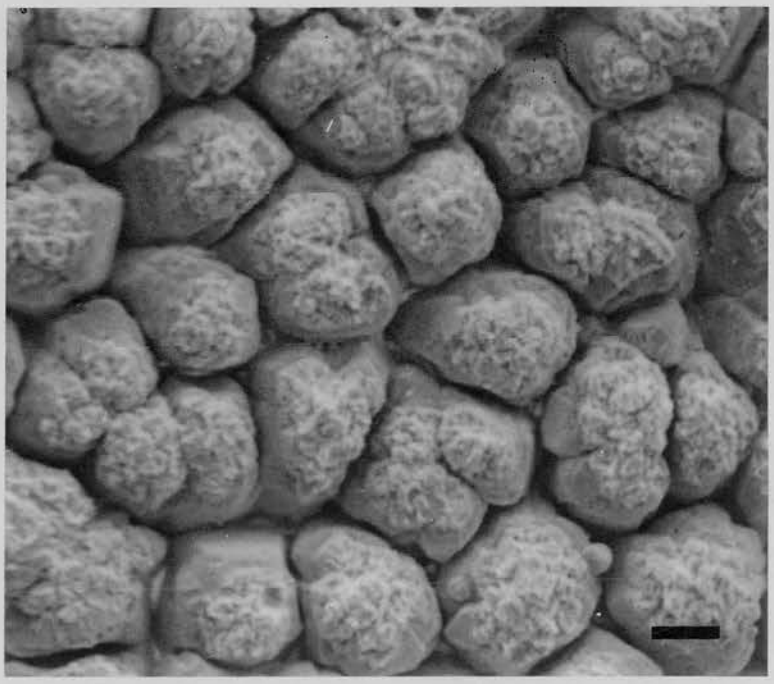

Figure 4. *The egg shells from hens treated with the lowest dosage of alendronate (experiment 1, group 3) exhibit minor modifications in the mammillary layer which are within the range of 'normality'. (Bar = 31 μm.)*

communication); therefore, to maximize the accuracy of the colorimetric response the serum samples were diluted for the measurement of alkaline phosphatase.

Birds were killed 15 days from the start of treatment, and the free thoracic vertebra and proximal tarsometatarsus fixed in buffered neutral formalin.

In the second experiment, 20 Hisex pullets were housed in individual laying cages. The pullets were 16 weeks of age. They were fed a standard layer diet and water *ad libitum*. The pullets were randomly divided into two groups (A and B) of ten. Twice a week the birds in group A were injected with 1 ml/kg of sterile water containing alendronate (0.01 mg/ml). The pullets in group B were injected with sterile water and served as a control. Injections were made subcutaneously over the superficial pectoral muscle. The pullets were killed when they had layed one egg and the proximal tarsometatarsus was fixed in buffered neutral formalin. The fixed bone samples from both experiments were processed to prepare undecalcified bone sections that were stained with toluidine blue, Mason Goldners trichrome or haematoxylin and eosin. The bone sections were examined blind and histomorphometry was used to quantify the percentages of medullary and trabecular bone (Wilson *et al.*, 1992).

The results of the experimental protocols were calculated and presented as a mean ± SD and analysed using Student's *t*-test.

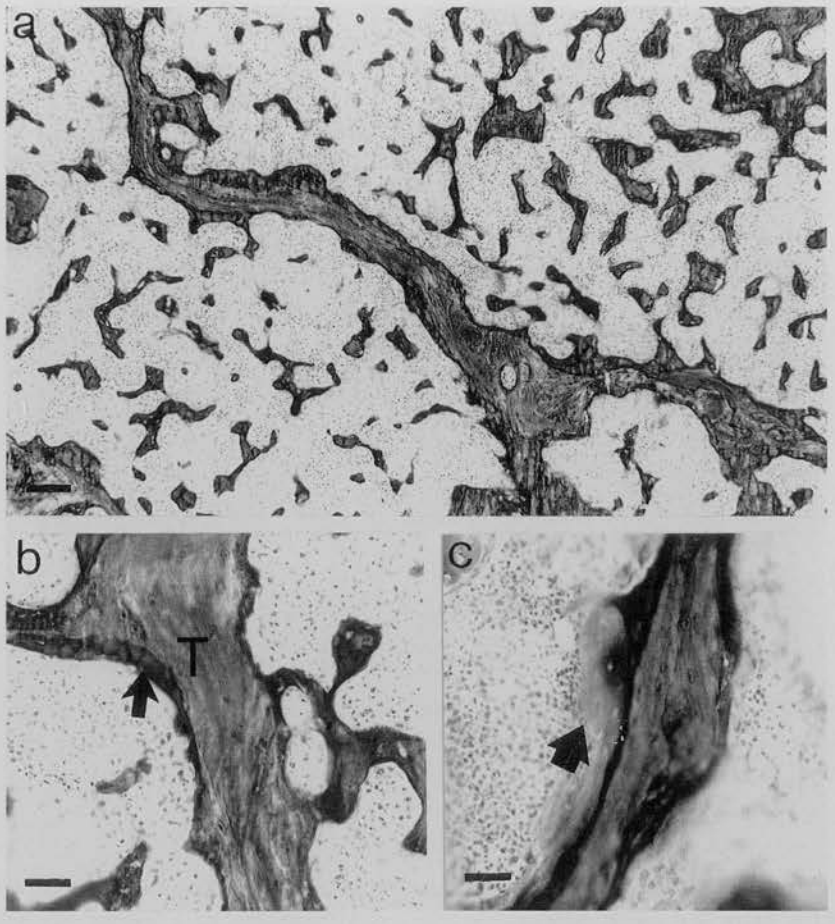

Figure 5. *(a) Tarsometatarsus from a bird receiving the highest dosage of alendronate (experiment 1, group 1), which had ceased laying and had no follicular activity (toluidine blue, bar = 50 μm). (b) In addition to trabecular bone (T), medullary bone (arrow) is still present (toluidine blue, bar = 12 μm). (c) Tarsometatarsus from a bird in group 2 with osteomalacia characterized by grossly thickened unmineralized seams of osteoid (arrow) on the medullary bone (toluidine blue, bar = 12 μm.)*

RESULTS

Experiment 1

Gross pathological examination revealed some calcification of the soft tissues and oedema in the subcutaneous tissues. These changes were only seen at the site of injection and only occurred in the hens injected with the most concentrated solution of alendronate (group 1; 1.0mg/kg). When the ovaries of the group 1 birds were examined, there was no evidence of follicular activity. The ovaries of the birds in the other three groups all had ovaries which showed apparently

Table 1. *Bone volumes of hens after treatment with alendronate for 2 weeks in mid-lay*

	Bone volumes (%)			
	Free thoracic vertebra		Prox tarsometatarsus	
Group[a]	Trabecular	Medullary	Trabecular	Medullary
1	10.67 (± 1.06)	2.07 (± 0.78)	10.30 (± 1.62)	8.04 (± 4.19)[c]
2	9.65 (± 1.49)	2.22 (± 0.77)	10.24 (± 1.80)	9.56 (± 3.06)
3	9.83 (± 2.88)	2.93 (± 0.83)	11.98 (± 1.95)	8.01 (± 3.25)[b]
4	8.62 (± 2.28)	2.07 (± 0.53)	9.51 (± 4.69)	12.46 (± 1.77)

[a]Groups 1, 2 and 3 were injected with 1.0, 0.1 and 0.01 mg/kg of alendronate, respectively, every second day. Group 4 birds were injected with sterile water.
[b]$P < 0.05$; [c] $P < 0.06$.

normal follicular activity. Marek's disease was present in one hen from group 2. There were no gross pathological changes noted in the hens of groups 3 and 4.

All the hens on the highest dosage of alendronate (group 1) stopped laying eggs by day 6, having laid a mean of 4.8 eggs.

The mean number of eggs laid by the controls (group 4; 11 eggs) was not significantly different from the mean number of eggs laid by the birds on the two lower dosages of alendronate groups 2 and 3; 12.5 and 12.4 eggs, respectively).

Serum calcium and alkaline phosphatase levels in the birds receiving the highest dose of alendronate (group 1) were significantly ($P < 0.05$) reduced compared to the controls (group 4). There was no significant change in plasma calcium or alkaline phosphatase levels of groups 2 and 3 compared to the controls. Changes in serum phosphate levels were not significant.

In the eggs from the controls (group 4) variations in the size of individual bodies were seen within the mammillary layer. The cap regions were deeply etched with the tracks formerly occupied by the membrane fibres (Figure 1). This strong bond between the organic and inorganic fractions of the shell provides a firm foundation for the formation of subsequent layers. The early fusion of the palisade columns also increases the effective thickness of the shell (Bain, 1991). Structural variation within the mammillary layer suggested a dose response to treatment with alendronate.

The egg shells from group 1 (Figures 2 and 3) displayed a variety of structural

Table 2. *Bone volumes of pullets after laying their first egg*

	Control[a]	Alendronate-treated[b]
Age (days)[c]	127.11 ± 4.7	131.3 ± 7.6
Trabecular bone volume (%)	16.7 ± 3.3	21.4 ± 4.1[d]
Medullary bone volume (%)	3.1 ± 1.3	2.3 ± 1.4

[a]Injected with sterile water.
[b]Injected with 0.01 mg/kg of alendronate twice a week from 17 weeks.
[c]Mean age ± SD of pullets after laying their first egg.
[d]$P < 0.025$.

variations in the mammillary layer consistent with a reduction in shell quality. The presence of type A bodies which although making some contribution to the growth of the palisade layer, had minimum contact with the membrane fibres. There was late fusion of the palisade columns resulting in a reduction in the effective thickness of the shell. Poorly etched caps were are also found and indicate minimal membrane contact (Figure 3).

The shells from the birds of group 2 (Figure 4) had a more compact mammillary layer, which did contain many rounded type B bodies. These structures fail on two accounts: their lack of membrane contact and their failure to contribute to palisade layer formation. The minor modifications observed in the mammillary layer of group 3 (Figure 4) are within the range of 'normality'.

Undecalcified histological sections showed slender metaphyseal trabeculae. In most birds the medullary bone was uniformly stained and the surface was covered in a layer of osteoid that varied in thickness. No osteoid was visible on the trabecular bone. It was noted that medullary bone was still present in the group 1 birds (Figure 5a and 5b), although they had ceased laying and had no follicular activity.

Osteomalacia, a reduction in bone mineralization as apposed to osteoporosis which is a reduction in bone volume, is characterized in laying hens by grossly thickened seams of medullary bone osteoid and poorly mineralized bone matrix. Osteomalacia was identified in the bone samples (Figure 5c) from two birds, one from group 1 and the other from group 2.

When tarsometatarsi from the three groups treated with alendronate were compared to the controls (group 4) (Table 1) there was a significantly ($P < 0.05$) lower volume of medullary bone volumes in group 3 and a reduction in volume approaching significance ($P < 0.06$) in group 1. In the free thoracic vertebrae, medullary bone was confined to the subchondral zone and was completely absent from some specimens that were highly pneumatized. Due to this variability the medullary bone of the free thoracic vertebrae could not be reliably used for statistical analysis. In this experiment in laying hens, alendronate had no significant effect on trabecular bone volumes.

Experiment 2

The results are shown in Table 2. There was no significant difference in the age at which the pullets in the two groups layed their first egg. The pullets treated with the alendronate had a significantly ($P < 0.025$) greater trabecular bone volume than the untreated controls. The lower mean volume of medullary bone seen in the alendronate treated pullets was not significant.

DISCUSSION

The present study indicated that alendronate has pharmacological activity in birds, and can alter bone volumes, plasma calcium levels and egg shell quality in laying hens. In pullets, alendronate administered during the onset of follicular activity resulted in a greater trabecular bone volume when the first egg was laid.

These observations may be of value in developing a regime that will reduce the incidence and severity of osteoporosis in hens.

Ultrastructural changes to the egg shells from the hens treated with alendronate in mid-lay indicated a dose-response to the drug. The highest dosage of alendronate resulted in structural abnormalities of the egg shells and a marked reduction in the serum calcium levels. The reduction in serum calcium may have reduced calcium availability for shell formation. This hypothesis is supported by the 'normal' egg shell morphology seen in the birds treated with the lowest dosage of alendronate, and in which there was no depression of serum calcium.

In the fowl there is a cyclical change in serum alkaline phosphatase levels that parallel the egg-laying cycle. Alkaline phosphatase levels were reduced in those birds that received the highest dosage of alendronate. These birds had stopped laying, were not mobilizing skeletal calcium for shell formation and medullary bone formation would have ceased. The reduction in osteoblast activity is likely to account for the drop in alkaline phosphatase in this group.

The tarsometatarsi of the mid-lay hens showed a significant decrease in medullary bone volumes, but there were no significant changes in the trabecular bone volumes. The statistical analysis of the first experiment may have been hampered by the large standard deviations in the groups which reflects the wide variation in trabecular and medullary bone volumes previously reported (Wilson & Duff, 1991; Wilson *et al.*, 1992).

Osteomalacia in laying hens is a response to a dietary deficiency of calcium (Wilson & Duff, 1991). Focal osteomalacia can occur in mammals due to diphosphonate treatment (Boyce *et al.*, 1984), but has not been attributed to alendronate administration. In the first experiment two hens had osteomalacia. These birds were in the groups given the highest dosages of alendronate. Osteomalacia is likely to be due to a failure of mineralization caused by a reduction in calcium availability. In these hens osteomalacia is attributed to a combination of the effects of a high dosage of alendronate and the calcium demands of egg shell formation.

Birds that stop laying, but still have follicular activity have much reduced medullary bone volumes, and those with no follicular activity have no medullary bone (Wilson & Duff, 1991). In the first experiment, despite the absence of follicular activity, medullary bone was seen in the group 1 birds. This may be a consequence of the very high dosage of alendronate severely inhibiting the removal of medullary bone.

In man, osteoporosis can be considered the result of inefficiencies in bone remodelling that results in a small bone deficit persisting on each completion of the resorption/formation cycle (Marcus, 1991). In the laying hen a complete sequence of medullary bone remodelling occurs during each egg-laying cycle. The marked increase in medullary bone resorption during shell formation is due to a rapid change in osteoclast activity (Miller, 1981), whereby osteoclasts on medullary bone are rapidly activated by parathyroid hormone (Miller, 1984). Nothing is known of the ability of osteoclasts to distinguish medullary and trabecular bone. In the laying hen, besides osteoclastic resorption of medullary

bone, there is a steady decline in trabecular bone volume through lay. Without trabecular bone formation the cumulative trabecular bone loss results in osteoporosis.

The results of experiment 2 indicate that when pullets are treated with alendronate before egg-laying, at the onset of lay there is a significant increase in trabecular bone volume. In pullets, between the onset of ovarian follicular activity and laying the first egg there is a marked reduction in trabecular bone volume (S. Wilson, manuscript in preparation). This loss of trabecular bone coincides with the initial formation of medullary bone. The mechanism of action of the alendronate is likely to be by reducing trabecular bone resorption during this period. In man, osteoporosis can be prevented by the maximization and maintenance of peak bone volume (Bhalla, 1993). Similarly, the prevention of early bone loss in egg-laying hens may be an important means of reducing the incidence and severity of osteoporosis.

The present study indicates that alendronate has a potential role in preventing trabecular bone loss in egg-laying hens. Bisphosphonates are maintained in the skeleton, there is little turnover of trabecular bone during lay. Alendronate treatment at the start of lay may help preserve trabecular bone throughout lay. Medullary bone formation and resorption during lay are unlikely to be compromised by alendronate treatment before lay, because new bone deposition subsequent to bisphosphonate treatment (i.e. medullary bone) should be normally resorbed.

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REFERENCES

BAIN, M. (1991). *A reinterpretation of eggshell strength. Egg and eggshell quality* (Ipswich, Wolfe Publishing Ltd).

BHALLA, A. K. (1993). Genetics, peak bone mass and early intervention in preventing osteoporosis. *Osteoporosis Review*, **1,** 1–3.

BLOOM, M., DOMM, L., NALBANDOV, A. & BLOOM, W. (1958). Medullary bone of laying hens. *American Journal of Anatomy*, **102,** 411–444.

BOYCE, B., SMITH, L., FOGELMAN, I., JOHNSTON, E., RALSTON, S. & BOYLE, I. (1984). Focal osteomalacia due to low-dose diphosphonate therapy in paget's disease. *Lancet*, **i,** 821–824.

ELOMMA, I., BLOMQUIST, C., GROHN, P., PARKKA, L., KAIRENTO, A., SELANDER, K., LAMBERG-ALLARDT, C. & HOMSTRON, T. (1983). Longterm controlled trial with diphosphonate in patients with osteolytic bone metastisis. *Lancet*, **i,** 146–149.

FLEISH, H. (1987). Bisphosphonates-history and experimental basis. *Bone*, **8,** Suppl. 1, 523–528.

GREGORY, N. & WILKINS, L. (1989). Broken bones in domestic fowl: handling and processing damage in end of lay battery hen. *British Poultry Science*, **30,** 555–562.

MARCUS, R. (1991). Skeletal ageing; Understanding the functional and structural basis of osteoporosis. *Trends in Endocrinology and Metabolism*, **March/April,** 53–58.

MEULLER, W., SCHRAER, R. & SCHRAER, H. (1964). Calcium metabolism and skeletal dynamics of laying pullets. *Journal of Nutrition*, **84,** 20–26.

MILLER, S. (1977). Osteoclast cell-surface changes during the egg-laying cycle in Japanese quail. *Journal of Cell Biology*, **75**, 104–118.

MILLER, S. (1981). Osteoclast cell-surface specializations and nuclear kinetics during egg-laying in Japanese quail. *American Journal of Anatomy*, **16**, 35–43.

MILLER, S. (1984). Morphology and ultrastructural aspects of the activation of medullary bone osteoclasts by parathyroid hormone. *Anatomical Record*, **208**, 223–231.

MINAIRE, P., BERNARD, E., MEUNIER, P., EDOUARD, C., GOEDERT, G. & PILOCHERY, G. (1981). Effects of disodiumdichloromethylene diphosphonate on bone loss in paraplegic patients. *Journal of Clinical Investigations*, **68**, 1086–1092.

REID, J. (1983). The use of the plasma chemistry unit as an aid to the scanning electron microscope study of avian eggshell structure. *British Poultry Science*, **24**, 233–235.

RIDDELL, C., HELMBOLT, C., SINGSEN, E. & MATTERSON, L. (1968). Bone pathology in birds affected with cage layer fatigue. *Avian Diseases*, **12**, 285–296.

SATO, M. & GRASSER, W. (1990). Effects of Bisphosphonates on isolated rat osteoclasts as examined by reflected light microscopy. *Journal of Bone and Mineral Research*, **5**, 31–40.

SEEDOR, J., QUARTUCCIO, H. & THOMPSON, D. (1991). The bisphosphonate alendronate (MK-217) inhibits bone loss due to ovarioectomy in rats. *Journal of Bone and Mineral Research*, **6**, 339–346.

SHINODA, H., ADAMEK, G., FELIX, R., FLEISH, H., SCHENK, R. & HAGEN, P. (1983). Structure-activity relationships of various bisphosphonates. *Calcified Tissue International*, **38**, 87–99.

SIMKISS, K. (Ed.) (1967). *Calcium in Reproductive Physiology*. (London, Chapman and Hall).

THOMPSON, D., SEEDOR, J., WEINREB, M., ROSINI, S. & RODAN, G. (1990). Aminohydroxybutane bisphosphonate inhibits bone loss due to immobilization in rats. *Journal of Bone and Mineral Research*, **5**, 279–286.

WILSON, S. & DUFF, S. (1991). The effects of vitamin or mineral deficiency on the morphology of medullary bone in laying hens. *Research in Veterinary Science*, **50**, 216–221.

WILSON, S., DUFF, S. & WHITEHEAD, C. (1992). The effects of age, sex and housing on the trabecular bone of laying strain domestic fowl. *Research in Veterinary Science*, **53**, 52–58.

RESUME

Effet chez la poule d'un bisphosphonate sur le volume des os et la structure de la coquille de l'oeuf

Les bisphosphonates utilisés pour la prévention et le traitement de l'ostéoporose chez l'homme peuvent prévenir les pertes osseuses dans les modèles expérimentaux de l'ostéoporose chez les mammifères. Chez les pondeuses, les fractures osseuses dues à l'ostéoporose sont très fréquentes. L'alendronate, un bisphosphonate, a été administré à trois groupes de poules en milieu de ponte. Chaque groupe a reçu une dose différente d'alendronate, le groupe 4 servait de groupe témoin. Les sujets ont été sacrifiés après 2 semaines de traitement. Les poules qui ont reçu la plus forte dose d'alendronate (1 mg/kg tous les 2 jours) ont cessé de pondre et leur concentration de calcium sérique a été réduite. Les poules qui ont reçu des doses plus faibles d'alendronate (0.1 et 0.01 mg/kg tous les 2 jours) présentaient une production d'oeufs et des concentrations de calcium sérique normales. Des coquilles d'oeufs, ayant des particularités ultrastructurales caractéristiques des coquilles de moindre qualité, ont été produites par des poules traitées avec les deux doses les plus élevées. Les coquilles des poules témoins et celles des poules ayant reçu la plus faible dose étaient considérées comme normales. Lorsque l'alendronate a été administré aux poules en milieu de ponte, aucun effet sur le volume de l'os trabéculaire n'a été observé, cependant le volume de l'os médullaire moyen a diminué dans certains groupes.

Lors d'une deuxième étude, des poulettes ont été traitées à l'alendronate (0.01 mg/kg deux fois par semaine) avant le début de la ponte. Les poulettes ont été sacrifiées après avoir pondu leur premier oeuf. Chez les poulettes traitées à l'alendronate, le volume de l'os trabéculaire était nettement plus élevé en début de ponte.

ZUSAMMENFASSUNG

Der Einfluß eines Biphosphonats auf das Knochenvolumen und die Eischalenstruktur bei der Henne

Biphosphonate, bei der Prophylaxe und Behandlung der Osteoporose beim Menschen verwendet, können Knochenverlust in Osteoporose-Versuchsmodellen bei Säugern verhüten. Bei legenden Hennen gibt es häufig Knochenbrüche, die auf Osteoporose zurückzuführen sind. Alendronat, ein Biphosphonat, wurde drei Hennengruppen in der Mitte der Legeperiode verabreicht. Den Gruppen wurden unterschiedliche Alendronatdosen verabreicht, und Gruppe 4 diente als Kontrolle. Die Tiere wurden nach zwei Behandlungswochen getötet. Die Hennen, die Alendronat in der höchsten Dosierung erhielten (1 mg/kg alle 2 Tage), hörten auf zu legen und hatten verminderte Serumkalziumspiegel. Bei niedrigeren Alendronatdosen (0.1 und 0.01 mg/kg alle 2 Tage) blieben Legeleistung und Serumkalziumspiegel normal. Eischalen mit ultrastrukturellen Besonderheiten, die eine verminderte Schalenqualität anzeigen, wurden von Hennen mit den zwei höheren Dosierungen gebildet, aber die Eischalen von den Kontrollen und von den mit der niedrigsten Dosis behandelten Hennen wurden für normal gehalten. Wenn Alendronat an Hennen in der Mitte der Legeperiode verabreicht wurde, hatte es keinen Einfluß auf das Volumen der trabekulären Knochen, aber in manchen Gruppen gab es eine Reduzierung des mittleren Volumens der Markknochen.

In einem zweiten Versuch wurden Junghennen vor Legebeginn mit Alendronat behandelt (0.01 mg/kg zweimal wöchentlich). Die Junghennen wurden getötet, nachdem sie ihr erstes Ei gelegt hatten. Bei den mit Alendronat behandelten Junghennen führte das zu einem signifikant vergrößerten Volumen der trabekulären (strukturellen) Knochen zur Zeit des Legebeginns.

RESUMEN

Efecto de un biofosfonato en el volumen óseo y en la estructura de la cáscara de huevo de la gallina ponedora

Los biofosfonatos utilizados en la prevención y tratamiento de la osteoporosis humana pueden prevenir la pérdida de hueso producida en modelos experimentales de osteoporosis en los mamíferos. Existe una gran incidencia de fracturas óseas en las gallinas ponedoras que son debidas a un cuadro de osteoporosis. Se administró alendronato, un biofosfonato, a tres grupos de gallinas ponedoras en la mitad de la puesta. Se administraron diferentes concentraciones de alendronato a cada grupo y se empleó el grupo 4 como control. Se sacrificaron las aves a las dos semanas del comienzo del tratamiento. Las gallinas que habían recibido la dosis máxima de alendronato > 1 mg/kg a intervalos de dos días) dejaron de poner y presentaron unas concentraciones reducidas de calcio sérico. Dosis inferiores de alendronato (0.1 y 0.001 mg/kg cada dos días) no alteraron la producción huevera normal ni las concentraciones de calcio sérico. Las aves que recibieron las dos dosis más elevadas de biofosfonato presentaron ultraestructuralmente signos de una reducción en la calidad de la cáscara de los huevos producidos, considerándose normales las cáscaras de los huevos procedentes de las aves control y de las aves que recibieron la dosis más baja. La administración de alendronato durante la puesta no afectó al volumen del hueso trabecular pero hubo una reducción en el volumen medular óseo medio en algunos grupos.

En un segundo experimento, se trataron pollitas con alendronato (0.01 mg/kg dos veces a la semana) antes del comienzo de la puesta. Las pollitas fueron sacrificadas tras el comienzo de la puesta. Las aves tratadas con alendronato mostraron un incremento significativo del volumen de hueso trabecular (estructural) al comienzo de la puesta.